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(54) **Title:** STABILIZING PROTEINS USING IONIC LIQUIDS

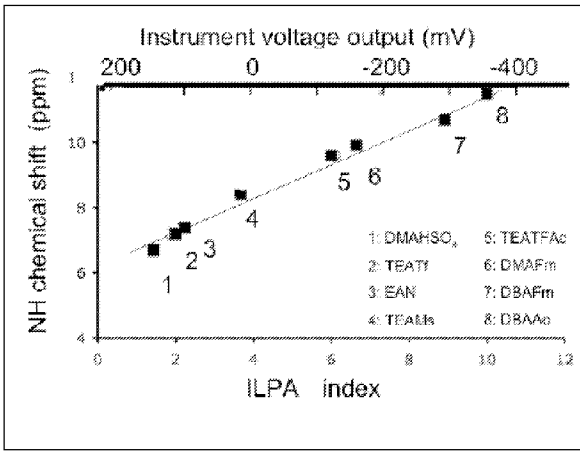

Figure 6. Linear variation of the proton activity (ILPA index) with N-H proton chemical shift (NMR) for 8 protic ILs at 25°C [11].
1. dimethylammonium hydrogen sulfate ($DMAHSO_4$)
2. triethylammonium triflate (TEATf)
3. ethylammonium nitrate (EAN)
4. triethylammonium methane sulfonate (TEAMs)
5. triethylammonium trifluoroacetates (TEATFAc)
6. dimethylammonium formate (DMAFm)
7. dibutylammonium formate (DBAFm)
8. dibutylammonium acetate (DBAAc)

FIG. 6

(57) **Abstract:** Among other things, techniques and compositions for stabilizing proteins are disclosed. To stabilizing a protein, lyophilized protein is dissolved in a low-viscous solvent that includes water and ionic liquid. Compositions and techniques relate to reversible folding-unfolding, aggregation protection and multi-year stabilization in high protein concentration solutions of protic and/or aprotic ionic liquids.

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STABILIZING PROTEINS USING IONIC LIQUIDS

CLAIM OF PRIORITY

[0001] This application claims priority under 35 USC §119(e) to U.S. Patent Application Serial No. 60/951,886, filed on July 25, 2007, the entire contents of which are hereby incorporated by reference.

BACKGROUND

[0002] This application relates to protein stabilization.

[0003] A protein molecule can be represented as unbranched chain of amino acids that can assume a specific three dimensional shape, the native state, to perform its biological function. Protein folding is the physical process by which protein molecules fold into their native states. Studies of protein folding can be carried out in buffered dilute aqueous solutions to minimize loss of protein to the aggregation phenomenon. There is a proton activity range (which for biological solutions can be described in terms of pH) in which the folded protein is most stable.

SUMMARY

[0004] This application describes, among others, compositions and techniques related to stabilizing proteins using ionic liquids. In particular, compositions and techniques related to reversible protein folding, unfolding and refolding, including techniques of providing reversible folding-unfolding, aggregation protection and multi-year stabilization in high protein concentration solutions of protic and/or aprotic ionic liquids are disclosed.

[0005] In one aspect, stabilizing a protein includes dissolving lyophilized protein in a low-viscous solvent that includes water and ionic liquid.

[0006] Implementations can optionally include one or more of the following features. Proton activity of the solvent can be adjusted to optimize stability of the protein. Dissolving the lyophilized protein can include dissolving the lyophilized protein in a non-viscous solvent that includes water and ionic liquid selected to optimize stability of the protein. Selecting the ionic liquid can include selecting a pure ionic liquid of a particular type to obtain an optimum proton activity. Selecting the ionic liquid can include selecting a mixture of two or more ionic liquids to obtain an optimum proton activity. In addition, the solvent can be controlled in a condition to stabilize the protein. Proton activity of the solvent can be detected using a device different from a standard pH meter. Proton activity of the solvent can be

measured using nuclear magnetic resonance chemical shift of N-H proton on a cation formed by proton transfer to a standard base.

[0007] In another aspect, stabilizing a protein includes dissolving lysozyme into a solvent that includes ethylammonium nitrate and water.

[0008] Implementations can optionally include one or more of the following features. The lysozyme can be dissolved into the solvent that includes 80 wt% ethylammonium nitrate and 20 wt% water. Lysozyme can be dissolved into a solvent having proton activity selected based on an ionic liquid proton activity (ILPA) index.

[0009] Yet in another aspect, a solution for stabilizing a protein includes a solute that includes a lyophilized protein; and a solvent that includes water and ionic liquid.

[0010] Implementations can optionally include one or more of the following features. The ionic liquid can include a protic type ionic liquid. The protic type ionic liquid can include one or more protic type ionic liquids selected to obtain an optimum proton activity.

[0011] Yet in another aspect, a solvent for stabilizing a protein includes ethylammonium nitrate and water.

[0012] Implementations can optionally include one or more of the following features. The solvent can include 80 wt% ethylammonium nitrate and 10 wt% water.

[0013] Yet in another aspect, stability of a protein is measured by generating an index as a percentage of the protein that refolds after a denaturing scan to temperatures beyond thermal denaturation. Generating the index includes measuring a denaturing enthalpy while upscanning the protein to a temperature above room temperature. Generating the index also includes cooling the protein to room temperature. Generating the index further includes measuring another denaturing enthalpy by rescanning the protein; and defining the index as a function of the two denaturing enthalpies.

[0014] Implementations can optionally include one or more of the following features. Upscanning to the temperature can include upscanning to 100 degrees Celsius. Defining the index can include defining the index as a fraction of the other denaturing enthalpy over the denaturing enthalpy.

[0015] The solutions and techniques as disclosed in this specification can be used in ways that provide one or more advantages. For example, a solution containing ionic liquid can support protein concentrations of 200 mg/ml. The solution containing ionic liquid can minimize or prevent water crystallization on cooling or reheating. The solution containing ionic liquid can prevent or minimize changes in the temperature of denaturation of proteins (e.g., an enzyme such as lysozyme. The solution containing ionic liquid can also prevent or minimize aggregation of either folded or unfolded protein and stabilize folded protein in the ambient

temperature liquid state over long periods of time. The conditions of stability for a given protein can be optimized. Further, the solution containing ionic liquid can if desired provide for total vitrification.

[0016] The stability of a protein in an ionic liquid solution of the type described in the foregoing can be assessed from the ability of the protein to refold after a thermal denaturation, which is a property that can be assessed rapidly, as described herein. The ability to refold (for a standard experimental protocol) should be a guide to long term stability for at least the following reasons. The unfolding event is a fluctuation about the equilibrium state which has a calculable probability (even at ambient temperature), once the energetics of unfolding are known (e.g. from the calorimetric studies). The probability of an unfolded molecule being present is obtained from the Privalov analysis of unfolding as a two-state chemical equilibrium. Aggregation and misfolding events, which proceed through the unfolded state, should therefore become more frequent the closer the system is to its Td. Thus the establishment of the refolding fraction, described herein, as a measure of stability is an important aspect of the present disclosure.

[0017] Among other things, the subject matter as described in this specification can be implemented as compositions of matter (e.g., solvents that confer enzyme stability), a method of manufacturing the compositions of matter, and method of

using the compositions of matter, and a method of assessing rapidly the efficacy of a solvent for conferring enzyme stability.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIG. 1 illustrates three successive denaturing cycles using a solution containing ionic liquid.

[0019] FIG. 2 shows progressive loss of protein to aggregation in solution with ionic liquid and without ionic liquid.

[0020] FIG. 3 illustrates the viscosities of various solutions with and without ionic liquid.

[0021] FIG. 4 illustrates a result of differential scanning calorimetry (DSC) upscan of lysozyme solutions with ionic liquid.

[0022] FIG. 5a illustrates DSC upscan of lysozyme solutions containing TEAMS with and without sucrose.

[0023] FIG. 5b shows a comparison of denaturation enthalpy with and without sucrose.

[0024] FIG. 6 illustrates a linear relation between proton activity and N-H proton chemical shift.

[0025] FIG. 7 shows refoldability fraction defined by a ratio of an area under the DSC unfolding endotherms of second to first upscans after change of ILPA index (insert shows unfolding endotherms in the most stable domain compared with those on either side.

[0026] FIG. 8 shows an example process 800 for stabilizing proteins.

[0027] FIG. 9 shows another example process 900 for stabilizing protein.

[0028] FIG. 10 shows an example process 1000 for measuring stability of a protein.

[0029] Like reference symbols and designations in the various drawings indicate like elements.

DETAILED DESCRIPTION

[0030] Techniques and materials are described for stabilizing protein using ionic liquid. Refolding of lysozyme in a solvent containing ionic liquid, after denaturation, in samples of lysozyme concentration up to 200 mg/ml is observed. At this concentration not only is refolding observed, but the process can be repeated many times with only small (≈3%) losses of refoldable sample per cycle, as judged by the enthalpy absorbed during the denaturation process. The small losses can be determined by comparison of the enthalpy absorbed during an initial unfolding scan with the energy absorbed in a second unfolding scan. This protocol is adopted because the differential scanning calorimetry (DSC) instrument is calibrated for HEATING runs. This measurement is an important part of the preservation assessment.

[0031] Various details on these measurements and the nature of the solutions that render this phenomenon observable are disclosed. The solutions developed using the techniques as disclosed in this specification can preserve the folded state of the protein (e.g., lysozyme) at room temperature in the liquid state, with losses < 20% per year for at least three years. The essential solution ingredient that is responsible for this behavior includes "ionic liquid." The term "ionic liquid" is currently applied to substances composed only of ions that remain in the liquid state below the boiling point of water, preferably down to room temperature. Many of these

exist and are currently the center of intense research activity, mainly as non-volatile solvents for chemical syntheses but also as ambient temperature liquids for electrochemical devices, heat transfer, and other purposes.

[0032] An unexpected stability of the protein cytochrome c can result when the protein is supported in solutions of a high ionic liquid content. The (non-toxic) ionic liquid of either aprotic or protic type can be used. For example, ionic liquid of aprotic type, with T_m = 114 °C, can be used, when a small amount of water is included. Also, a ionic liquid of a protic type (e.g., ethylammonium nitrate (EAN), with T_m = 13 °C) can be used to confer stabilization of proteins. The solutions as disclosed in this specification can also include a sugar, but the addition of sugar is not needed to confer protection against aggregation in the liquid state, as described in detail below.

[0033] Ultimately, various conditions for achieving the remarkable and unexpected stabilization of the protein in ionic liquid containing solvents can be optimized. Such optimization is obtained by adjusting the proton activity or effective pH of the supporting solution. Understanding the basis for this stabilization has been impeded by absence of suitable techniques for quantifying the characteristics of the supporting media, in particular the effective pH, since these liquids bear little relation to the systems for which pH is defined.

[0034] The techniques and systems described in this specification implement an index of proton activity using nuclear magnetic resonance (NMR). For example, the proton activity index can be implemented using an NMR chemical shift of the nitrogen-hydrogen (N-H) proton on a cation formed by proton transfer to a standard base, such as triethylammine, or diethlmethylamine. The smaller the shift from the reference (Tetramethylsilane) the smaller the effective pH. This chemical shift is shown with respect to FIG. 6 below.

[0035] Such NMR based index of proton activity can be used to measure the effectiveness of the solutions that provide the protection mentioned above. Such protective solutions can be developed for various purposes including providing a medium in which protein refolding could be studied at low temperatures in the absence of any interference from the crystallization of ice. In one aspect, the formulation of such protein solution can be designed to eliminate the possibility of nucleation of ice crystals without eliminating water from the solution. Then the energy release during folding of the protein at low temperatures, after an initial quench had preserved the unfolded state, can be observed free of any exothermic energy releases due to ice formation.

[0036] Proteins are often stabilized in the glassy state by use of sugars, trehalose being found of special effectiveness. For example, a medium in which to observe refolding of quenched denatured states can be created using such solutions

(i.e., a sugar such as trehalose). However, using such a sugar solution, the fraction of unfolded protein that could be observed to refold during warm-up after initial quench is quite small, about 0.2. The refolding fraction rises dramatically when an equal mass fraction of EAN, $[CH_3CH_2NH_3^+][NO_3^-]$, is added to the solution. This addition of EAN also greatly decreased the viscosity. A very satisfactory solution composition, which supported a protein (e.g., lysozyme concentration of 200 mg/ml) with about 97% protection against aggregation in a single unfold-refold cycle, includes the following weight percentages of sugar, ionic liquid, water and protein: sucrose 27 wt%, EAN 31 wt%, water 20 wt%, lysozyme 22 wt%. In some implementations, the sugar is omitted, and the composition could be described as protein 40 wt %, EAN 40 wt % and water 20 wt%.

[0037] The concentration of the protein enzyme, lysozyme, in the above solution is sufficient to give a very clear quantifiable endotherm during upscans at 20 °C/min using a standard DSC, differential scanning calorimeter, TA 2920, or Perkin-Elmer DSC-7. Three successive denaturing cycles are shown in Fig. 1, where it is seen that the endotherms decrease in area by about 3% per cycle. For the above solution, the temperature of the endotherm maximum, 74 °C, and the enthalpy absorbed, are the same as reported for the normal in-vitro (aqueous buffer) process. The denaturation temperature is sensitive to the sugar/EAN ratio. The quantitative assessment

of the area under the endotherm is readily obtained with the instrument software which applies a standard baseline, and this is compared with that of the initial scan after each of ten cycles. Results are shown in Fig. 2. A systematic, decelerating, decrease is observed with successive cycles. The loss rate is strikingly reduced from that observed on cycling a solution that did not contain the EAN, shown by the dashed curve in Fig. 2. (Experimental uncertainty, indicated by vertical bar, is greater in this case.).

[0038] The sugar-EAN-water-protein solution as described above also avoids having a high viscosity. The viscosities of some sugar-EAN-water solutions in the absence of the protein can be only about an order of magnitude greater than that of water. This viscosity is sufficiently low that the viscous retardation of molecular processes in the refolding of the protein is not a significant effect. The viscosity-temperature relations for some solutions of interest are compared with that for water in Fig. 3.

[0039] In some implementations, sugars other than sucrose, such as glucose can be used. Under certain conditions, equally satisfactory results are obtained using glucose as the sugar in the solution, which gives a somewhat less viscous solution (Fig. 3). Also, Trehalose can be used to provide equivalent protection to a certain extent. However, using trehalose can generate a heat absorbing anomeric exchange that appears at about the same temperature as the unfolding

endotherm, which can reduce the effectively of trehalos for energy studies by making the studies more complicated. In some implementations, the solution can be implemented without any types of sugar.

[0040] The manner in which the different energetic steps of the folding process of lysozyme can be followed, during warm-up from the glassy state obtained by quenching after denaturation, can also be measured. Further, the ionic liquid solution can stabilize proteins, such as lysozyme, against deterioration is described. Previously stored samples are rescanned after storage (e.g., for various periods of time, which can span several years) in (a) a phial in a refrigerator, and (b) at room temperature in sealed aluminium DSC pans, during the period of storage. Some samples are stored for up to three years. The scans, taken using a TA instrument at 20 °C/min, are shown in Fig. 4 where the unfolding enthalpies per g protein are noted on the figure.

[0041] The unfolding enthalpy in the case of the refrigerated sample is quite unchanged from freshly prepared samples. The sample stored at room temperature has a smaller unfolding enthalpy, about 1/3 that of the fresh sample, which may imply that 2/3 of the folded protein has been lost to aggregation or hydrolytic decomposition since encapsulation, i.e. about 22% per year. However, this is a worst-case number. This previously stored sample had been used in cycling experiments. The composition and sample mass are known but the number of

cycles it experienced is not recorded. Based on the Fig. 2 evaluation, at least 20% of the sample may have been lost during the cycling experiments, and this is not included in assessing the annual loss rate. Protein samples under normal laboratory study in buffered aqueous solutions are typically only stable to exposure to ambient temperatures for hours, so it is clear that the ionic liquid has bestowed an extraordinary level of protection on the protein. Lysozyme is, reputedly, a fairly "tough" protein, however, so the preservation potential of EAN may be modified for less robust proteins.

[0042] Concerning refoldability, not all proteins behave as lysozyme in FIG. 1. While ribonuclease does, neither myoglobin nor cytochrome c refolds after hot denaturation in concentrated solutions (though apo-myoglobin does). Cytochrome c also does not refold, though it can not refold in pH 4 aqueous solutions when oxidized and dilute enough. On the other hand, lysozyme hot refolds in solutions containing alternative ionic liquids of effective pH similar to EAN, both with and without a sugar component. The sugar component may be helpful only to enhance the glass-forming properties that are desirable for the energetics-of-refolding studies.

[0043] Figs. 5a and 5b show the aggregation resistance of lysozyme during repeated unfold-refold cycles in the two solvents, triethylammonium methane sulfonate (TEAMS) 29 wt% + sucrose 30 wt% + water 19 wt% + lysozyme 22 wt%, that differ

only in the presence or otherwise of sucrose. TEAMS is low melting, Tm = 21.6 °C. Some ~100 other protic ILs are also available.

[0044] Fig. 5(a) shows that the unfolding temperature in TEAMS, which has a slightly lower effective pH than the EAN-based solvent, is a little higher than in biological solutions, and it is also a little higher in unfolding enthalpy. Both may provide a more stable folded structure. Using the solutions as disclosed in this specification, the effect of the increased stability on the refolding energy pathways can also be measured. As seen in Fig. 5(a), when the sugar is removed from the solutions, the denaturation occurs at distinctly lower temperatures, however, as seen in Fig. 5(b) the resistance to aggregation is not affected. Thus, the solutions as disclosed in this specification can further be used to measure the effect on the cold refolding energetics. In addition, the solutions as disclosed in this specification can be used to measure the effect of larger changes in the effective pH that can be induced by alternative choices of ionic liquid.

[0045] Hence, a method of stabilizing protein based on the information in this specification can include the following: (1) dissolve lyophilized protein in water-ionic liquid non-viscous solution to make 1:1 solution; (2) add on part of glucose or sucrose; (3) establish baseline using solution with folded protein, quenched to glassy state and reheated,

recording; (4) heat to denaturation and requench to glass; (5) second heating run to observe refolding, and second unfolding, recording; and (6) repeat for consistency check.

[0046] Various conditions can be optimized to achieve an unexpected stabilization of protein dissolved in supporting media that contains ionic liquid. For example, the conditions for obtaining protein stabilization can be optimized by adjusting the proton activity or "effective pH" of the supporting media. Adjusting the proton activity includes one or more of the following: (1) adjusting the type of ionic liquid (e.g., aprotic or protic) used; (2) combining different ionic liquids having different Ionic Liquid Proton Activity determined by N-H proton chemical shifts or (ILPA) indexes (a full description of ILPA indexes is provided below); (3) adjusting a composition of a mixture of ionic liquids of different pure-substance ILPA index; and (4) changing the water content of the supporting media. For example, large water contents tend to reduce stability. The ILPA index may have a greater impact to the protein stability than the actual water content in the supporting media. In this specification, protein stability is characterized based on a "refoldability index" for illustrative purpose and other parameters may be used to characterize protein stability either independently from the refoldability index or in a combination with the refoldability index.

[0047] In applications of protic or aprotic ionic liquids that contain trace amount of water, the activity of protons are adjusted to optimum values. To quantify the proton activity in ionic liquid containing media, a suitable means (e.g., apparatus) is developed. After large voltage calibrations, standard pH meters that respond to concentration of hydronium ions can be used to obtain readings that vary with anion and cation types in ionic liquids. However, such standard pH meters saturate at readings around the pH neutral point. In addition, standard pH meters show an erratic relation to other direct probes of the state of protons, such as the N-H chemical shift. Further, the meaning of "pH" measured by such standard pH meter devices is problematic when measuring ionic liquids. For example, while protic ionic liquids can have a concentration of protonated species (the analog of the H_3O^+ ion) of 5-10 molar, some ionic liquids can be strongly basic in character. The activities of the protons in ionic liquids can vary enormously depending on the nature of the acid and base from which the ionic liquids were formed.

[0048] The stability of a protein can be assessed without a waiting period of months or years. The observed protein stability can be quickly assessed in terms of the refolding fraction (or refoldability index), i.e. the fraction of the initial amount of protein that is observed by calorimetry to be unfolding after a prior thermal unfolding. For a standard experimental protocol, the ability of the protein to refold

provides a guide to the long term stability. The unfolding event can be represented as a fluctuation about an equilibrium state once the energetics of unfolding are known from the calorimetric studies, for example. The equilibrium state has a calculable probability even at ambient temperature. The probability of an unfolded molecule being present is obtained from the Privalov analysis of unfolding as a two-state chemical equilibrium. Aggregation and misfolding events, which proceed through the unfolded state become more frequent the closer a system is to its thermal denaturation (Td). Thus, the techniques and systems described in this specification provides for an establishment of the refolding fraction as a measure of protein stability.

[0049] To assess the stability of the protein by the refolding fraction, a refolding index (RFI) is provided as a standard of stability measurement. RFI is expressed as a percentage of an initial scan area obtained in the next scan immediately following the refolding on temperature decrease after first unfolding.

[0050] The RFI represents a percentage of the protein that refolds after a denaturing scan to temperatures beyond Td. To determine the RFI differential scanning calorimetry (DSC) is used. An initial denaturing enthalpy (ΔHd1) is measured during 20K/min upscan to 100° C. After cooling to room temperature at instrument rates (~20K/min from 100 °C), the sample is rescanned to obtain a second unfolding, yielding ΔHd2. The RFI

is then defined by RFI = (100ΔHd2/ΔHd1). For stable conditions, RFI is found to be 97-99, i.e. 97-99% of the protein refolded upon cooling to room temperature.

[0051] Misfolded states, in contrast to aggregated states, continue to contribute to the RFI because the misfolded states also unfold endothermically. The misfolded states are revealed by lower temperature components of the unfolding endotherm, hence by departures from simple two-state unfolding phenomenology.

[0052] The correlation of refolding ability with long-term stability can also be directly supported by the following observations. Samples of HWL in a PIL (hydroxyethylammonium nitrate) with RFI 97 measured 14 months after initial measurement and thereafter stored in vials at RT, yielded the same Td, ΔHd and RFI. However a sample (in 1:1 EAN: EAformate solution) whose RFI 14 months ago was only 50, yielded a Td 5°C lower and an unfolding enthalpy that is 15% lower, i.e. additional aggregation/hydrolysis deterioration occurred. Furthermore, the biological activity (using micrococcus lysodeikiticus cells observed at 450nm [8]) of the 14mth old RFI 97 sample was found it to be 91%, compared with that for a fresh sample, 92%.

[0053] For hen white lysozyme (HWL), the refolding fraction can be as large as 0.97 per cycle in the favorable proton activity domain. Comparable refolding fraction values can be obtained for ribonuclease A and α-lactalbumin. This stability

index (described in terms of refolding fraction or refoldability index) falls off quite rapidly on either side of a high stability range. Further, this specification describes the consequence of controlled excursions from the high stability regime in the cases of HWL, and its similarly structured protein cousin, lactalbumin.

[0054] In another aspect, to measure proton activity in ionic liquid media, a device is created to be sensitive to the proton binding that has a natural zero point within the spectrum of liquids that are fully ionic. In addition, a practical scale is provided for measuring Ionic Liquid Proton Activity as an ILPA index. Such device can be used to characterize the solutions that stabilize proteins, and to demonstrate that stability of proteins in ionic liquid containing media is a simple function of the ILPA index. The device and the ILPA index can be implemented according to the contents of Appendix B.

[0055] The device or instrument for measuring proton activity in ionic liquid media includes a modified version of a common glass electrode. The common glass electrode responds to the activity of protons in a test solution by detecting the movement of mobile ions (usually sodium) in a special glass membrane developed over a long period of research and development. Commercial pH electrodes are unreliable when measuring concentrated solutions. In particular, readings obtained from a commercial pH electrode can often be erratic

and can suffer from undesired saturation at levels beyond pH 7. This is due primarily to the aqueous solutions within the commercial pH electrode.

[0056] Thus, an ILPA meter is developed as a modified instrument of a standard pH meter. The ILPA meter contains an ionic liquid (e.g., triethylammonium triflate (TEATf)) within both the sensing component and the reference component. In contrast, aqueous solutions are found in the glass electrode of a commercial pH meter. By using ionic liquids, the instrument (ILPA meter) becomes sensitive to proton activity. Since a pH electrode is essentially a voltmeter, the ILPA meter is designed to measure the proton transfer energy between the Bronsted acid and Bronsted base pairs. The ILPA meter is calibrated in the common way, but large mV offsets are measured in ionic liquid media when compared to aqueous solutions. The effectiveness of this instrument can be judged from the linear relation between the measured voltage and another direct measure of the state of the protons in a protic ionic liquid, the N-H proton chemical shift, which is demonstrated in FIG. 6.

[0057] Sugar can be added to the supporting medium that contains ionic liquid to prevent ice formation during quench refolding. However, sugar is not a necessary component in the supporting medium for the purposes of stabilization. Thus, the supporting medium for optimizing protein stabilization as described in this specification can include minimum of two

variables for a chosen concentration of protein: (1) the type of ionic liquid used in the supporting media; and (2) the water-to-protein ratio.

[0058] Fig. 7 illustrates a variation of the folding fraction (refoldability index) for lysozyme with change of ionic liquid ILPA index. Note that the composition of the ionic liquid component is varied, at constant water content, within two binary mixtures (1) ethylammonium nitrate (EAN), weakly acidic -triethylammonium triflate (TEATf), highly acidic; and (2) EAN - dibutylammonium acetate (DBAAc), highly basic. Together, these binary mixtures cover a wide range of proton activities. Additional values are provided by other systems both within and outside the range of the two binaries. There is a rather broad proton activity zone around ILPA index 4-6 in which the refolding fraction is roughly constant at 0.97, with rapid fall-off on either side. This high refolding fraction does not depend on the presence of the "folding aid" EAN but rather is obtained with any PIL, pure or solution, that provides an ILPA index in the stable range. Similar results can be obtained when the water content is varied, changing the ILPA index. Again, a refolding maximum (i.e., optimizes protein stability) appears for the ILPA index range of 4-6. Thus, the proton activity rather than the actual water content or the actual ionic liquid composition, is identified as the important variable in optimizing stability of protein.

[0059] Reducing the refolding fraction (e.g., by changing the ILPA index) affects the enthalpy of the refolding process. As the high stability zone in Fig. 7 is exited, in either direction, the enthalpy of unfolding strongly decreases. Unlike the refolding fraction, however, the enthalpy of unfolding does not approach zero but rather levels off near the smaller values seen in the Fig. 7 insert. This is similar to the effect of pH on denaturation enthalpies (and "melting" temperatures) of lysozyme and other proteins in familiar aqueous solution systems.

[0060] FIG. 8 shows an example process 800 for stabilizing proteins. A lyophilized protein is dissolved in a low-viscous solvent that includes water and ionic liquid (802). The Lyophilized protein can be dissolved in a non-viscous solvent that includes water and ionic liquid selected to optimize stability of the protein, for example. A pure ionic liquid of a particular type can be selected to obtain an optimum proton activity. Also, a mixture of two or more ionic liquids can be selected to obtain an optimum proton activity. The proton activity of the solvent can be adjusted to optimize stability of the lyophilized protein (804). The solvent can be controlled in a condition to stabilize the protein (806). The proton activity of the solvent can be detected using a device different from a standard pH meter (808). The pH in the ionic liquid present solvent cannot be stably measured using a standard pH. For example, a customized glass electrode can be

implemented with ionic liquid used as an internal sensing solution. Also, the proton activity of the solvent can be detected by measuring the nuclear magnetic resonance chemical shift of N-H proton on a cation formed by proton transfer to a standard base.

[0061] FIG. 9 shows another example process 900 for stabilizing protein. Lysozyme is dissolved into a solvent that includes ethylammonium nitrate and water (902). For example, the lysozyme can be dissolved into the solvent that includes 80 wt% ethylammonium nitrate and 20 wt% water. Dissolving the lysozyme can include dissolving the lysozyme into a solvent having proton activity selected according to an ionic liquid proton activity (ILPA) index (904).

[0062] FIG. 10 shows an example process 1000 for measuring stability of a protein. An index is generated as a percentage of the protein that refolds after a denaturing scan to temperatures beyond thermal denaturation (1002). Generating the index includes measuring a denaturing enthalpy while upscanning the protein to a temperature above room temperature (1004). Upscanning to the temperature can include upscanning to high temperatures such as 100 degrees Celsius, for example. The protein is cooled to room temperature (1006). Another denaturing enthalpy is measured by rescanning the protein (1008). The index is defined as a function of the two denaturing enthalpies (1010). For example, the index can be

defined as a fraction of the other denaturing enthalpy over the denaturing enthalpy.

[0063] As described in this specification, protein stability can be optimized by using a method of controlling or adjusting the proton activity. The level of proton activity controls the ability of a common protein dissolved in supporting media containing one or more ionic liquids to refold after unfolding. The proton activity can be adjusted by changing either the ionic liquid or water content. By passing well beyond the stable conditions, a new low energy, soluble, unfoldable/refoldable state of lysozyme has been uncovered.

[0064] While this specification contains many specifics, these should not be construed as limitations on the scope of any invention or of what may be claimed, but rather as descriptions of features that may be specific to particular embodiments of particular inventions. Certain features that are described in this specification in the context of separate embodiments can also be implemented in combination in a single embodiment. Conversely, various features that are described in the context of a single embodiment can also be implemented in multiple embodiments separately or in any suitable subcombination. Moreover, although features may be described above as acting in certain combinations and even initially claimed as such, one or more features from a claimed combination can in some cases be excised from the combination,

and the claimed combination may be directed to a subcombination or variation of a subcombination.

[0065] Similarly, while operations are depicted in the drawings in a particular order, this should not be understood as requiring that such operations be performed in the particular order shown or in sequential order, or that all illustrated operations be performed, to achieve desirable results.

[0066] Only a few implementations and examples are described and other implementations, enhancements and variations can be made based on what is described and illustrated in this application.

CLAIMS

What is claimed is:

1. A method for stabilizing a protein comprising:

obtaining a low-viscous solvent that includes water and ionic liquid; and

dissolving a lyophilized protein in obtained low-viscous solvent.

2. The method of claim 1, further comprising adjusting proton activity of the solvent to optimize stability of the protein.

3. The method of claim 1, wherein dissolving lyophilized protein includes dissolving the lyophilized protein in a non-viscous solvent that includes water and ionic liquid selected to optimize stability of the protein.

4. The method of claim 3, wherein selecting the ionic liquid comprises selecting a pure ionic liquid of a particular type to obtain an optimum proton activity.

5. The method of claim 4, wherein selecting the ionic liquid comprises selecting a mixture of two or more ionic liquids to obtain an optimum proton activity.

6. The method of claim 1, further comprising controlling the solvent in a condition to stabilize the protein.

7. The method of claim 1, further comprising detecting proton activity of the solvent using a device different from a standard pH meter.

8. The method of claim 1, further comprising detecting proton activity of the solvent by measuring nuclear magnetic resonance chemical shift of N-H proton on a cation formed by proton transfer to a standard base.

9. A method of stabilizing a protein comprising:

obtaining a solvent that includes ethylammonium nitrate and water; and

dissolving a solute including lysozyme into the obtained solvent.

10. A method of claim 9, further comprising dissolving the lysozyme into the solvent that includes 80 wt% ethylammonium nitrate and 20 wt% water.

11. The method of claim 9, wherein the dissolving comprises dissolving lysozyme into a solvent having proton

activity selected according to an ionic liquid proton activity (ILPA) index.

12. A solution for stabilizing a protein comprising:

a solute comprising a lyophilized protein; and

a solvent comprising water and ionic liquid.

13. The solution of claim 12, wherein the ionic liquid comprises a protic type ionic liquid.

14. The solution of claim 12, wherein the protic type ionic liquid comprises one or more protic type ionic liquids that is selected to obtain an optimum proton activity.

15. A solvent for stabilizing a protein comprising:

ethylammonium nitrate; and

water.

16. The solvent of claim 15 further comprising 80 wt% ethylammonium nitrate and 20 wt% water.

17. A method of measuring stability of a protein comprising:

generating an index as a percentage of the protein that refolds after a denaturing scan to temperatures beyond thermal denaturation comprising:

measuring a denaturing enthalpy while upscanning the protein to a temperature above room temperature;

cooling the protein to room temperature;

measuring another denaturing enthalpy by rescanning the protein; and

defining the index as a function of the two denaturing enthalpies.

18. The method of claim 17, wherein upscanning to the temperature comprises upscanning to 100 degrees Celsius.

19. The method of claim 17, wherein defining the index comprises defining the index as a fraction of the other denaturing enthalpy over the denaturing enthalpy.

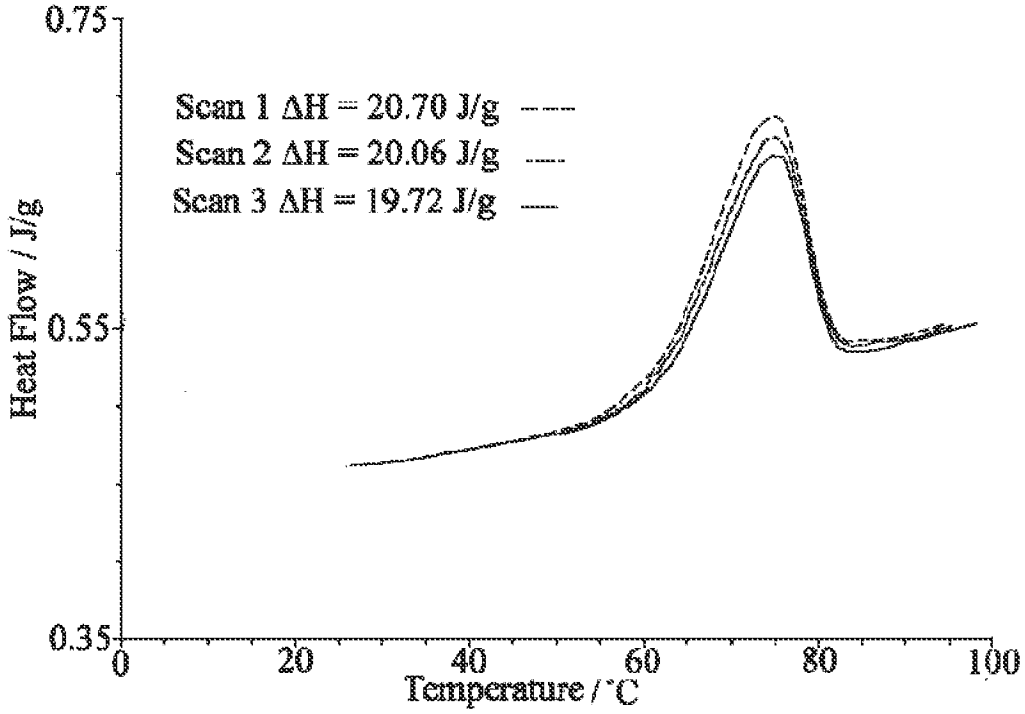

Fig. 1 Three successive denaturing scans with peak temperature at 74 °C, showing small (3% per cycle) loss of endotherm area from scan to scan.

FIG. 1

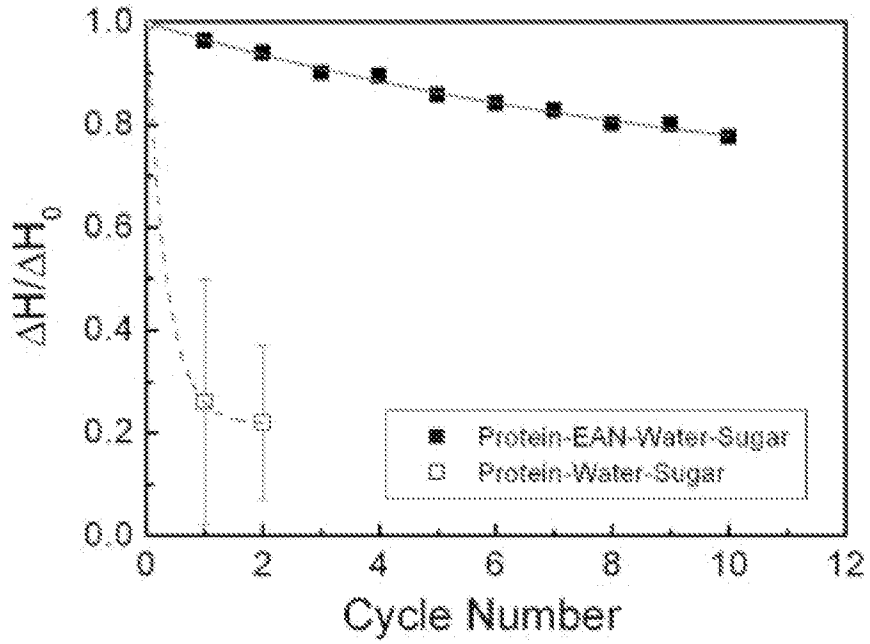

Fig. 2 Progressive loss of protein to aggregation over 10 cycles, assessed by endotherm area comparisons.

FIG. 2

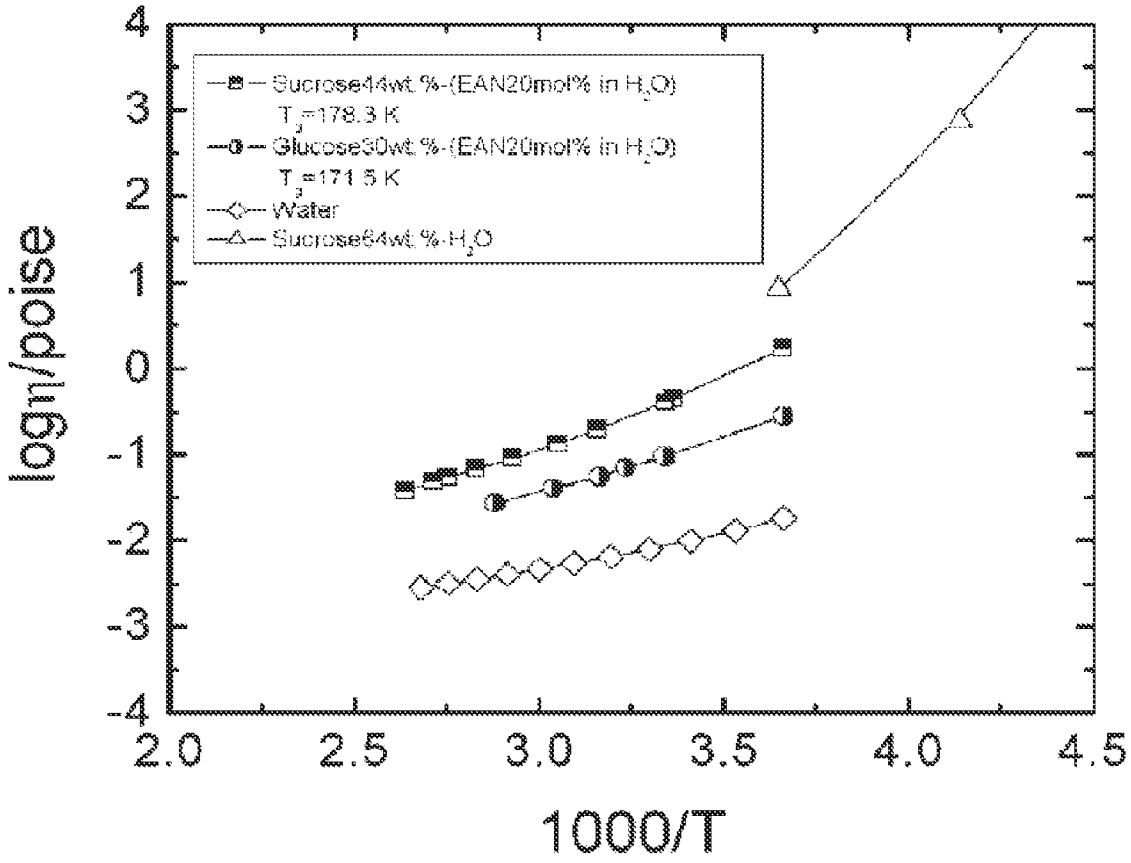

Fig. 3 Viscosity of 44% sucrose + 33% EAN + 22% water solvent (corresponding to 34.7% sucrose + 26.9% EAN + 17.8% water + 20.6% lysozyme solution) and 30% glucose + 42% EAN + 28% water solvent solutions, in the temperature range ambient to −100 °C. The viscosity of water (open diamond) is included for comparison.

FIG. 3

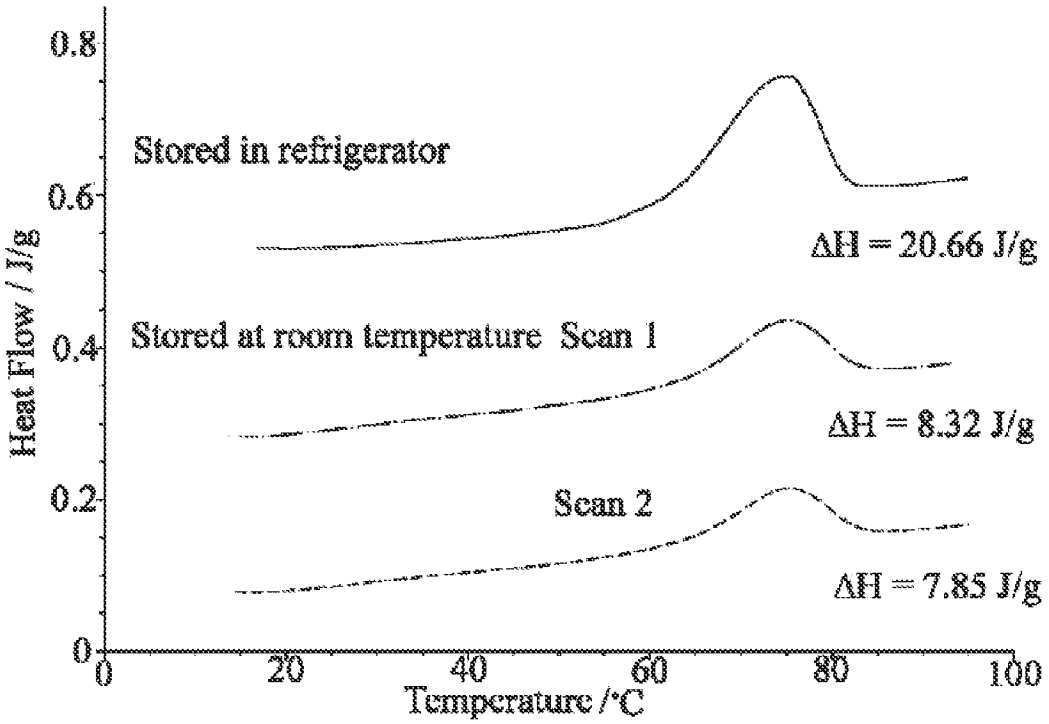

Fig. 4 DSC upscan of lysozyme solutions in EAN–sugar solutions after three years in (a) refrigerated sample, (b) DSC pan stored at room temperature. In the latter case a second scan is included to show that the smaller peak diminishes in size with unfold/fold cycling at a rate comparable to that of fresh samples. Much of the protein has survived over the years at ambient since the 2003 denaturation/renaturation studies.

FIG. 4

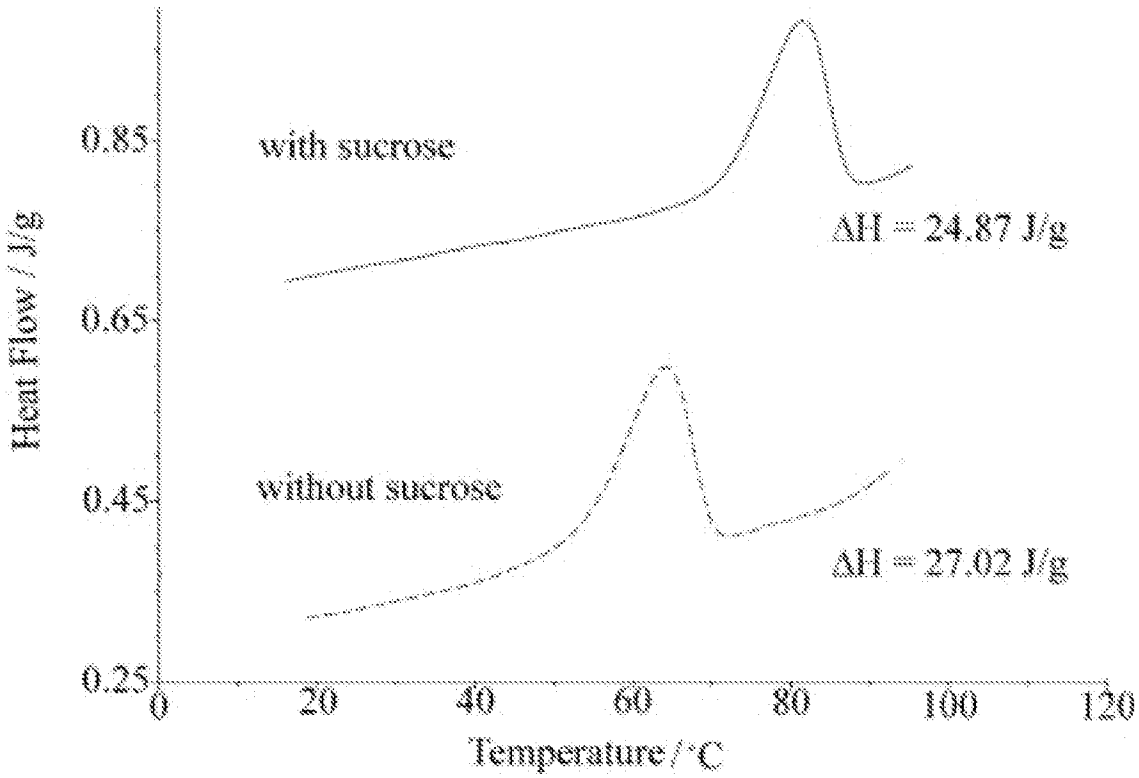
Heat Flow / J/g
0.85
0.65
0.45
0.25
with sucrose
ΔH = 24.87 J/g
without sucrose
ΔH = 27.02 J/g
0
20
40
60
80
100
120
Temperature / °C

FIG. 5a

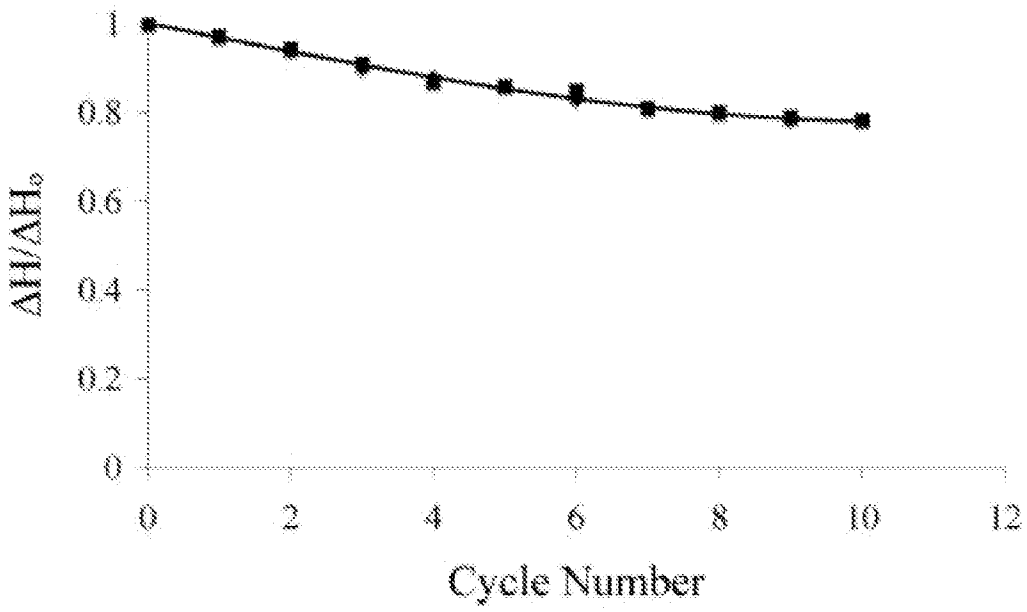

Fig. 5 (a) DSC upscans of lysozyme solutions containing TEAMS, with and without sucrose. Denaturation enthalpies are given in J per g protein. In the absence of sucrose, denaturation occurs below the temperature in aqueous buffer. Sucrose, and in particular trehalose, have been shown to increase the thermal stability (denaturation temperature) of proteins.[22,24] (b) Comparison of denaturation enthalpy with (■) and without (◆) sucrose in relation to cycle number. Behavior is comparable to that seen in Fig. 2. No sucrose effect on protein aggregation is seen, so protection against aggregation can be attributed to the 'ionic liquid'.

FIG. 5b

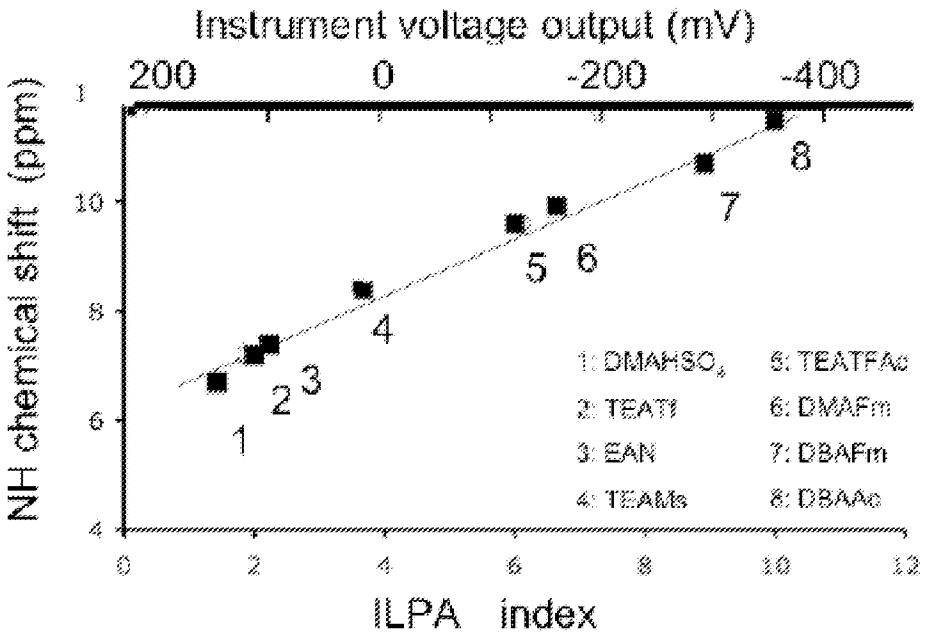

Figure 6. Linear variation of the proton activity (ILPA index) with N-H proton chemical shift (NMR) for 8 protic ILs at 25°C [11].

1. dimethylammonium hydrogen sulfate ($DMAHSO_4$)
2. triethylammonium triflate (TEATf)
3. ethylammionium nitrate (EAN)
4. triethylammonium methane sulfonate (TEAMs)
5. triethylammonium trifluoroacetates (TEATFAc)
6. dimethylammonium formate (DMAFm)
7. dibutylammonium formate (DBAFm)
8. dibutylammonium acetate (DBAAc)

FIG. 6

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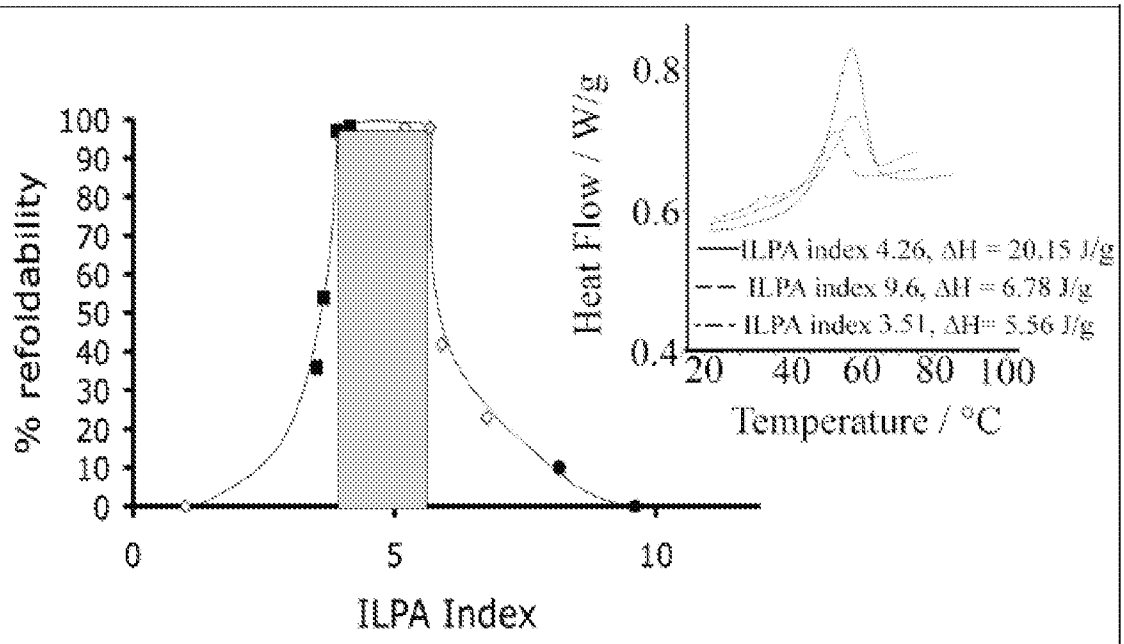

Figure 7. Refolding fraction (defined by the ratio of area under the DSC unfolding endotherms of second to first upscans after change of ILPA index. **Insert.** Unfolding endotherms in the most stable domain compared with those on either side (ILPA indexes 3.51 and 9.6). closed squares varying molare concentrations of EAN-TEATf, closed circles are neat DBAAc and 1:1 wt% EAN-DBAAc, open circles : ILPA index 1 , ethylammonium hydrogen sulfate, $EAHSO_4$, ILPA index 6.76 EAFm, ILPA index 6 EAN-EAFm 1:1 wt%, ILPA index 5.2 EAN-TEATFAc 1:1wt%.

FIG. 7

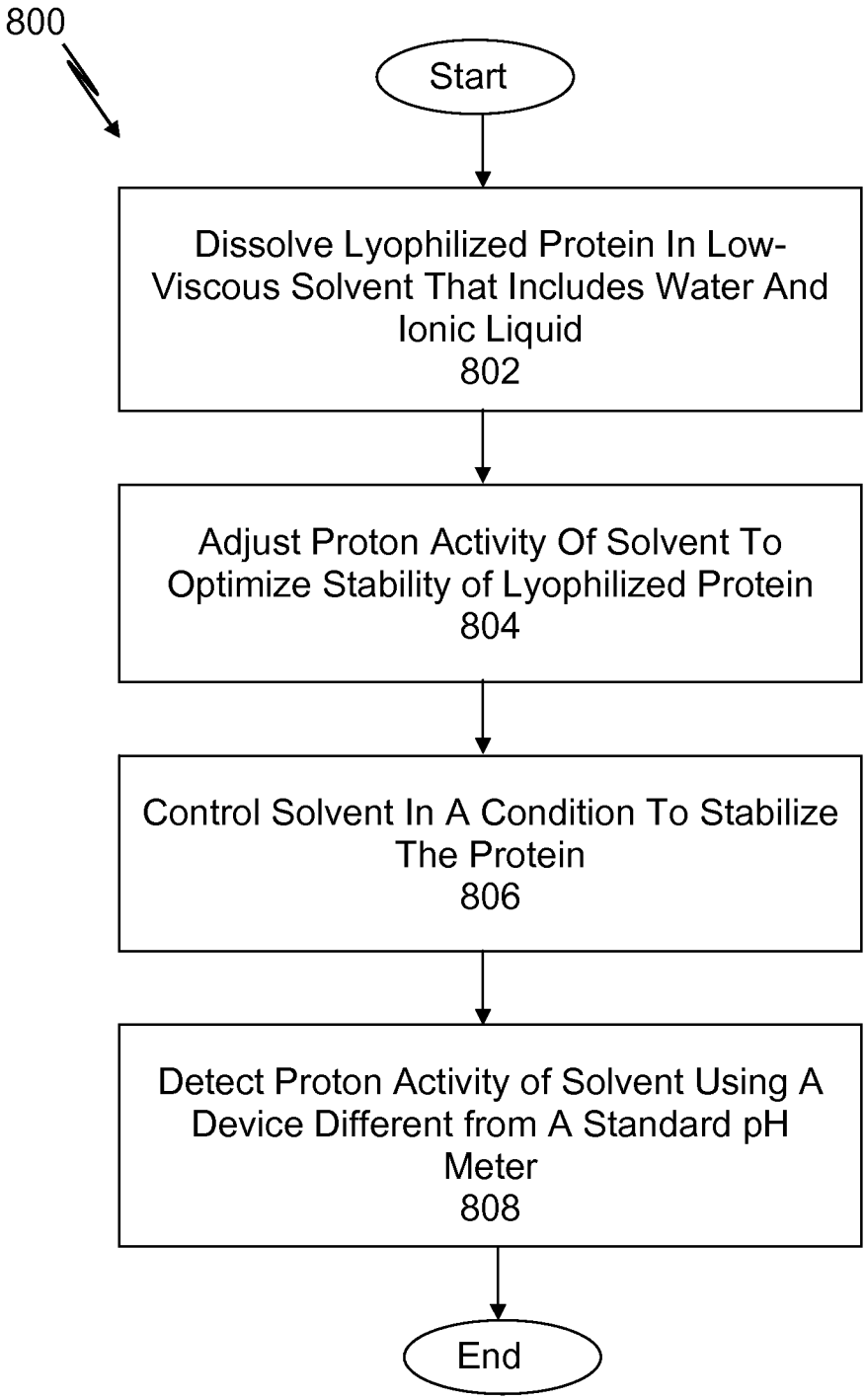
800
Start
Dissolve Lyophilized Protein In Low-Viscous Solvent That Includes Water And Ionic Liquid
802
Adjust Proton Activity Of Solvent To Optimize Stability of Lyophilized Protein
804
Control Solvent In A Condition To Stabilize The Protein
806
Detect Proton Activity of Solvent Using A Device Different from A Standard pH Meter
808
End

FIG. 8

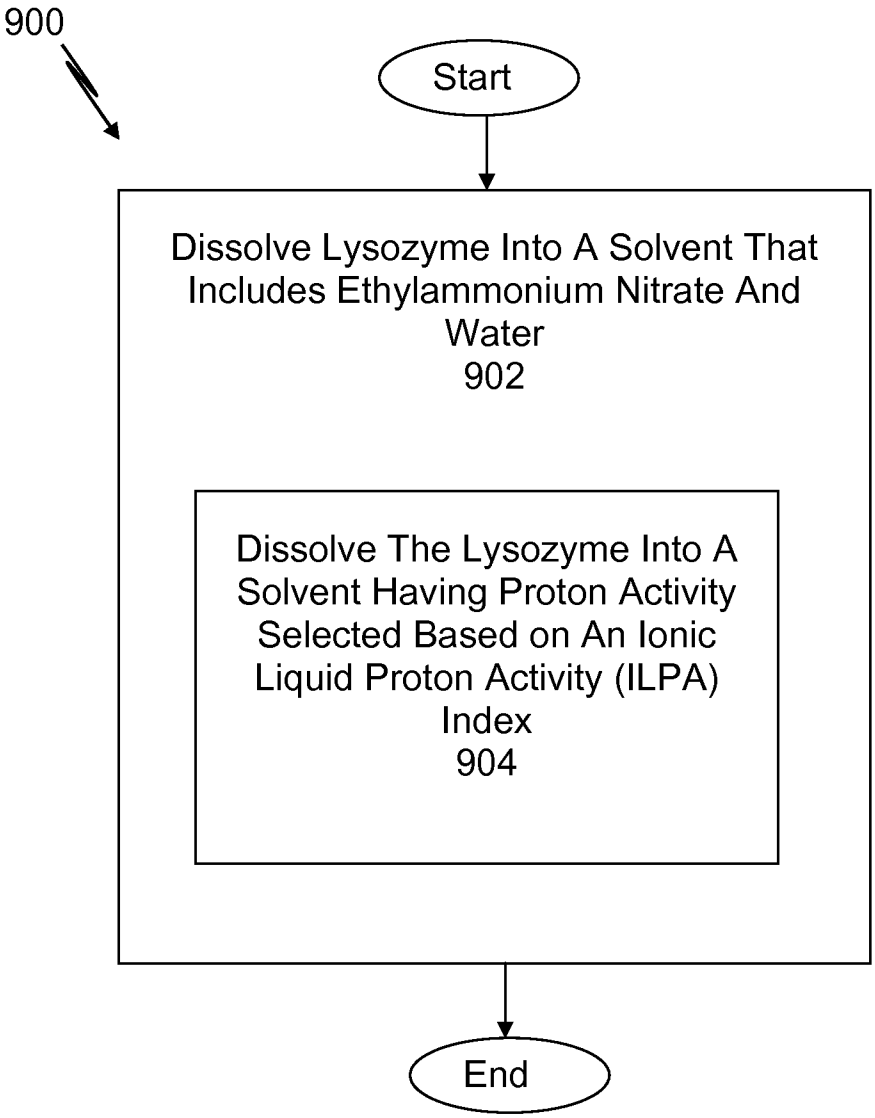
900
Start
Dissolve Lysozyme Into A Solvent That Includes Ethylammonium Nitrate And Water
902
Dissolve The Lysozyme Into A Solvent Having Proton Activity Selected Based on An Ionic Liquid Proton Activity (ILPA) Index
904
End

FIG. 9

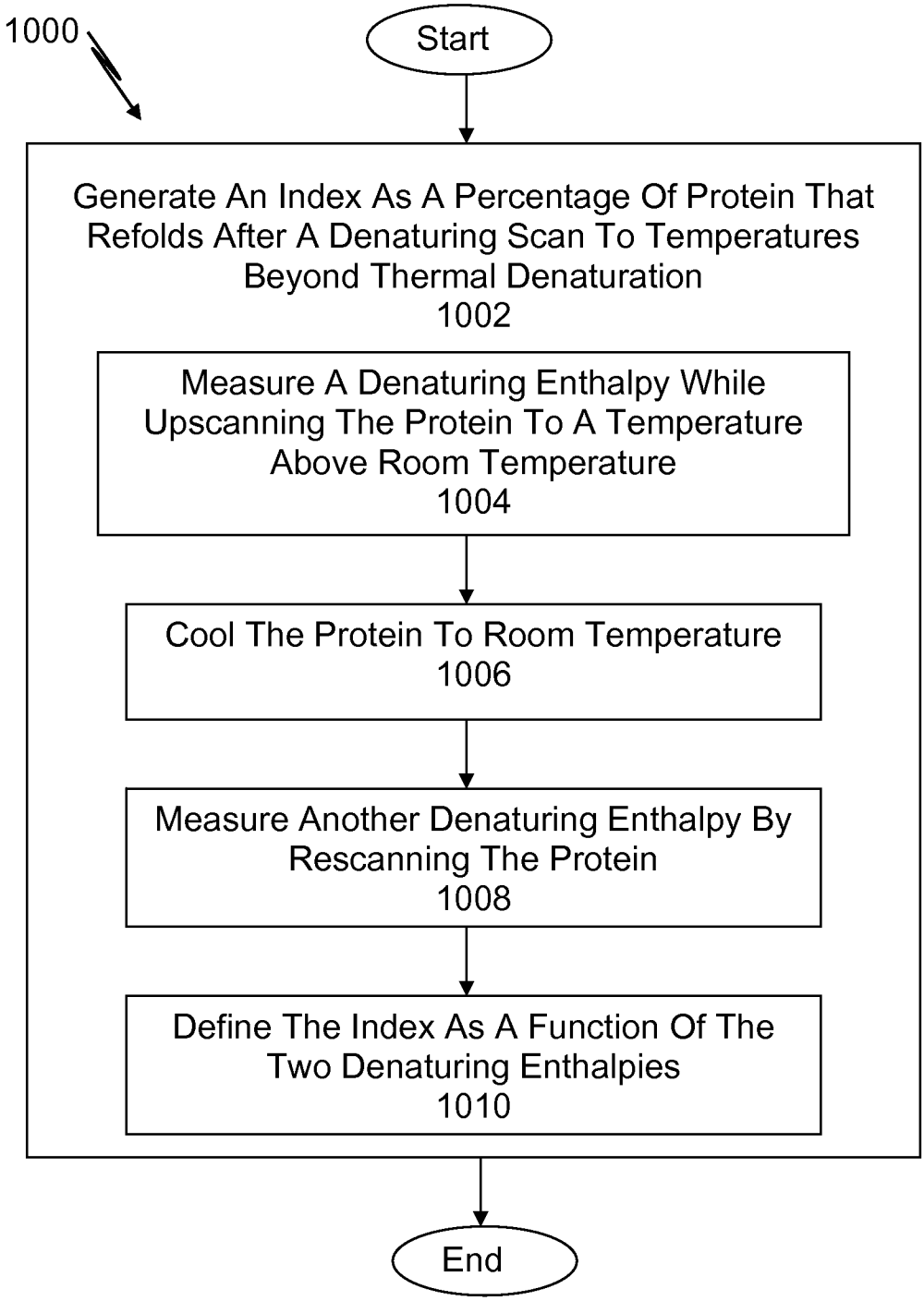
1000
Start
Generate An Index As A Percentage Of Protein That Refolds After A Denaturing Scan To Temperatures Beyond Thermal Denaturation
1002
Measure A Denaturing Enthalpy While Upscanning The Protein To A Temperature Above Room Temperature
1004
Cool The Protein To Room Temperature
1006
Measure Another Denaturing Enthalpy By Rescanning The Protein
1008
Define The Index As A Function Of The Two Denaturing Enthalpies
1010
End

FIG. 10