従来散剤と称されてきた造粒散剤同様に，一部軟膏剤に名称変更の必要性が生じることとなった．そこで対策として，販売名については，乳化しているもので「○○軟膏」としているものは「○○軟膏」と称してよい旨を当局からの通知で示すこととなった．また，局方各条収載のうちで，乳化している軟膏剤である「親水軟膏」は「親水クリーム」，「吸水軟膏」は「吸水クリーム」と名称が変更されたが，「親水軟膏」および「吸水軟膏」の名称も別名として残すこととした．

3 その他

a 経口投与製剤と口腔内適用製剤

いずれも口から投与する製剤であるが，経口投与製剤は消化管内で薬物が吸収される製剤であるのに対し，口腔内適用製剤は口腔内で薬物が吸収される製剤である．したがって放出性（溶出性）の条件などに大きな違いがあり，品質管理に用いる試験あるいはその条件は異なる．したがって別製剤として分類することとした．

b 口腔内崩壊錠

口腔内崩壊錠の利点として「水なしでも服用できる」ということがあげられ，その利点を記載すべきという意見も寄せられた．しかし水なしの服用は窒息事故の危険性も伴うため，口腔内崩壊錠であっても水を用いて服用することが望ましい．したがって「水なしでも服用可」とは記載しなかった．

c 経口ゼリー剤とゲル状の経口液剤

ゼリーは弾性のある一様な分散体で，水分を非常に多く含むものであり，容器から取り出した時にその形が保たれているものである．そこで経口ゼリー剤は「流動性のない成形したゲル状の製剤」と規定した．一方，経口液剤にも高分子増粘剤が添加されるゲル状の製剤があるが，このような製剤を含むように経口液剤は「液状又は流動性のある粘稠なゲル状の製剤である」と規定した．すなわち経口ゼリー剤とゲル状の経口液剤は流動性の有無で区別した．製剤試験としては，経口ゼリー剤の場合は溶出試験または崩壊試験の適用が求められる．

d 経皮吸収型製剤

経皮吸収型製剤は，日局15で1つの剤形として追加された．しかし，経皮吸収型製剤は，有効成分を全身循環血流に送達することを目的とした皮膚適用製剤の総称であり，1つの剤形として分類することは難しい．そこで日局16では，剤形として独立させず，11. 皮膚などに適用する製剤の項の(1)に，その定義を記載するとともに，その放出速度について「経皮吸収型製剤からの有効成分の放出速度は，通例，適切に調節される」と言及した．

e 生薬関連製剤

丸剤，酒精剤，芳香水剤は既存製品のほとんどが生薬を有効成分として含有する製剤であるので生薬関連製剤に分類した．また，現在でも使用されているということを理由に茶剤を復活させた．

製剤総則改正後の課題

日本薬局方は医療用医薬品，一般用医薬品，生薬含有製剤，薬局製剤，院内製剤すべてを対象としている．したがって，このたびの製剤総則改正はきわめて大きな改訂であり，製薬企業，医療関係者，関連研究機関などへの周知を図ることが重要である．また各条収載製剤についても，総則改正にあわせて，記載の整備を徹底することが必要である．

また大きな課題としては，一般試験法に記載のない製剤特性の試験法の設定がある．現在「適切な○○性を有する」としてある製剤特性（表2）について，可能なものから一般試験

表2 製剤各条において「本剤は，適切な○○特性を有する」とされた製剤特性のリスト

日局一般試験法に設定されていない試験法

- 崩壊性，溶出性：口腔内崩壊錠，口腔用錠剤
- 噴霧量の均一性*：口腔用スプレー剤，吸入粉末剤，吸入エアゾール剤，点鼻剤，スプレー剤
- 放出性：(1)埋め込み注射剤，持続性注射剤(放出性)；(2)座剤，膣錠，膣用坐剤，貼付剤**(放出特性)；(3)経皮吸収製剤**(放出速度)
- 粘　性：口腔用半固形剤，眼軟膏剤，直腸用半固形剤，軟膏剤，クリーム剤，ゲル剤
- 粘着性：貼付剤

*局方国際調和課題として取り上げられており，比較的早い段階で一般試験法の設定が可能と思われる製剤特性

**欧米の薬局方に収載されている一般試験法の導入が容易と考えられる製剤特性

法の整備が必要である．このなかには国際調和で取り上げられている試験法などがあり，近々に整備が可能と考えられる．

このたびの製剤総則改正の方針の1つは「国際的整合性」にあった．製剤の分類および定義，製剤試験内容については，欧米の動向をみながら改正を行った．しかし一方で欧米の薬局方も改正の議論を行っているところである．したがって，日本薬局方としても改正内容に関する情報発信を積極的に行い，欧米薬局方と齟齬ができないように注意を払う必要がある．

さらに，将来にわたっての課題としては，今後も医療現場において導入され標準的に用いられるようになった新規製剤については，速やかに製剤各条へ収載するよう，フォローアップが重要である．

おわりに

このたびの製剤総則改正は，半世紀ぶりともいえる大改正であった．

改正製剤総則では，臨床で標準的に使用されている製剤をほぼ網羅するとともに，投与部位および適用部位を大分類し，製剤の形状や特性などによりさらに細分類した．この改正により，医薬品製剤の全体像の把握が可能になり，医療関係者の医薬品製剤の教育用テキストとして使用することもできよう．そのうえで，各製剤の品質管理に必要な基本的な要件(製剤試験，容器・包装，貯法など)を整備したことにより，医薬品品質管理のテキストとしても使用できると思われる．

大きな改正のため，残された課題も少なくないが，日局16製剤総則の何よりの成果は，今後臨床現場に導入される製剤についても，速やかに収載を可能とする柔軟性のあるフレームワークの完成にあると考えている．

謝辞

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製剤試験法

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Key Points

- 日局16一般試験法製剤試験では国際調和試験法として日局15に取り込まれた試験法を中心に，部分的な改正が行われた．
- 多くは，日局15国際調和試験法についてその後の実態に即した修正，国際調和試験法の各局方への取り込みの際に生じた問題の修正である．
- その他，製剤総則大改正，水各条の改正に伴う記載整備がある．

はじめに

第十五改正日本薬局方(日局15)では製剤に関する一般試験法のなかで，製剤均一性試験法，注射剤の採取容量試験法，注射剤の不溶性微粒子試験法，崩壊試験法，溶出試験法について，国際調和試験法の取り込みが行われた．日局16では新たな国際調和試験法の収載はなく，また新規試験法の収載もなかった．一方，国際調和案の各局方への取り込みの確認作業のなかで，問題点などが見つかり，部分的改正がなされたほか，製剤総則の大改正，および水各条の改正に伴う記載整備のための改正がなされた．以下，試験法別に解説する．

眼軟膏剤の金属異物試験法

眼軟膏剤の金属異物試験は製剤総則中の眼軟膏剤の金属性異物を試験する試験法である．日局15においては，判定基準は製剤総則の眼軟膏剤各条中に記されていた．しかし，日局15第一追補以降，以下の判定基準は一般試験法の眼軟膏剤の金属異物試験法中の「3．判定」の項に記すこととした．

「本剤10個の50μm以上の金属性異物の合計数は50個以下であり，かつ個々の平底ペトリ皿のうち金属性異物が8個を超えるものが1枚以下のときは適合とする．これに適合しないときは，更に20個について同様に試験し，本剤30個の金属性異物の合計が150個以下であり，かつ個々の平底ペトリ皿のうち金属性異物が8個を超えるものが3枚以下のときは適合とする．」

製剤の粒度の試験法

日局15製剤総則では，顆粒剤と散剤は粒子径の違いで分類されていた．しかし日局16製剤総則は大改正され，粒子径による分類ではなく，造粒工程の有無により顆粒剤と散剤と

を分類することとした．ただし，顆粒剤のなかで，細粒および散と称してよい製剤については，粒子径で区別することとした．したがって，この判定に用いる粒度試験を除いて，試験法の記述は削除された．

注射剤の不溶性微粒子試験法

注射剤の不溶性微粒子試験は，混入してはならない不溶性微粒子を試験する方法であり，日局15で国際調和試験法に大改正された．その後国際調和試験法の取り込みについての確認作業において，日局15について国際調和試験法を十分反映していないという指摘が欧州薬局方からなされ，以下の2点について，国際調和試験法をより直接的に反映させた記載に改正した．

(1) 日局15の導入部分は，従来の日局一般試験法の記載にあわせて試験法の目的を述べる一文で始まっている．一方国際調和試験法では，不溶性微粒子の定義に始まっている．そこで，日局16では国際調和を尊重するために，国際調和試験法の記載にしたがって以下の通りの導入文に改正した：「注射剤（輸液剤を含む）の不溶性微粒子とは，これら製剤中に意図することなく混入した，気泡でない容易に動く外来性，不溶性の微粒子である．」

(2) 国際調和試験法では，微粒子試験用水をフィルター水と表現している．一方日局15では「微粒子試験用水」を試薬で定義し，不溶性微粒子数を規定した精製水としていた．日局16では，国際調和を考慮して，微粒子試験用水を「孔径0.45μm以下のメンブランフィルターを通した水で，自動微粒子装置を用いて測定した不溶性微粒子数は，10mL当たり10μm以上のもの5個以下，25μm以上のもの2個以下である」とした．

以上のほか，第2法顕微鏡粒子計測法の一般注意事項における微粒子試験用精製水の確認の部分が，日局15では編集上のミスで誤記載されていることがわかり，日局16では国際調和試験法に沿った記載に改めた．

点眼剤の不溶性微粒子試験法

点眼剤の不溶性微粒子試験は，製剤総則中の点眼剤の不溶性微粒子の大きさおよび数を試験する試験法である．日局15においては，点眼剤の不溶性微粒子試験法の判定基準は製剤総則の点眼剤各条中に記されていた．しかし，日局16局では以下の判定基準は一般試験法の点眼剤の不溶性微粒子試験法中の「3．判定」の項に記すこととした．

「本剤1mL中の個数に換算するとき，300μm以上の不溶性微粒子が1個以下であるときは適合とする．」

崩壊試験法

崩壊試験は錠剤，カプセル剤，顆粒剤などにおいて試験液中に定められた条件で規定時間内に崩壊するかどうかを確認する試験である．この試験に用いる試験器の補助板のサイズの記述について，装置の実態にあわせて，「台形の平行線の下端部は長さ1.6±0.1mmで円周部からの深さ1.6±0.1mmの位置にあり」を「台形の平行線の下端部は長さ1.6±0.1mmで円周部からの深さ1.5～1.8mmの位置にあり」に改正した．この改正にあわせて，崩壊試験装置（図1）も改正した．

溶出試験法

溶出試験は経口製剤の吸収速度に密接に関

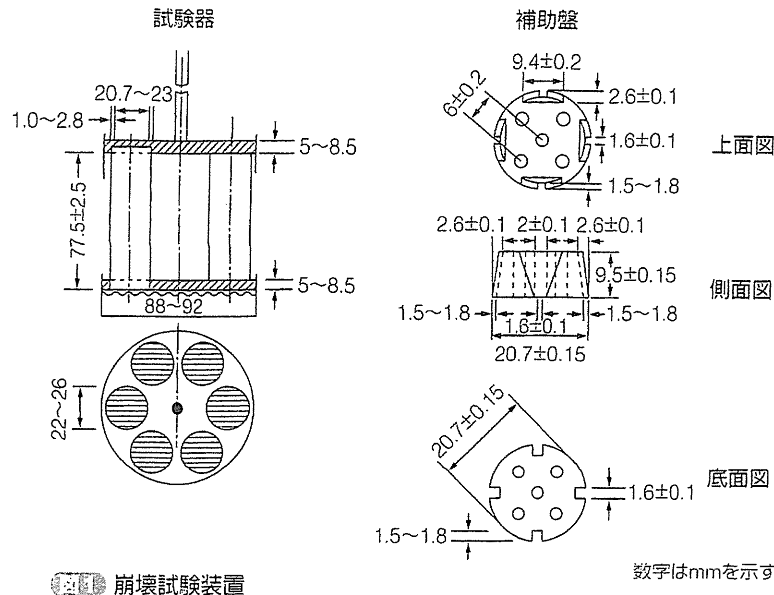

図1 崩壊試験装置

係する品質特性である溶出性を試験し，これら医薬品の製品の一定性確保のために行う試験法であるが，日本薬局方では併せて著しい生物学的非同等性を防ぐことも目的とした試験とされている．溶出試験法は日局15で国際調和され大きく改正されたが，日局16では以下の点について，部分的な改正が行われた．

1 パドル法で用いるシンカー

パドル法はわが国では溶出試験で標準的に使用されている方法であり，ベッセル中の溶出試験液に攪拌翼と回転軸からなるパドルを入れ，回転翼を回転し攪拌，試料から溶出試験液に溶出してくる有効成分を測定する方法である．その際，錠剤などの試料が浮く場合は，らせん状に数回巻いた針金のような科学的に不活性な材質でできた小型の締め付けないシンカーを試料に取り付けることができる．日局15ではわが国で使用されてきたシンカーのみでなく種々のシンカーが使用可能となった．しかし溶出試験結果はシンカーの形状により影響を受けるので，日局16では従来のシンカーと異なるものを用いる場合は，その形状などを記載することとして，「また，それら以外のバリデートされたシンカーを用いることもできる．シンカーを使用することが規定されている場合，シンカーは別に規定する」という一文を挿入する改正を行った．ただしこの改正部分は日局16独自の記載であり不調和部分である．

なお日局15のシンカーの仕様例の図(図2)は，らせん状の針金は5巻きでありピッチが3.0～3.5mmであるにもかかわらず，全長が25～26mmとされ，製造が不可能な例であったため，日局16では7巻半の例に図が改められた．

2 フロースローセル法の脈流

フロースローセル法は，送液用ポンプで試験液をフロースルーセルに送液し，セル中の試料から溶出する有効成分を測定する方法である．用いるポンプについては，日局15では「定流量(表示流量の±5%)で送液でき，脈流の波形は120±10パルスの正弦型でなけ

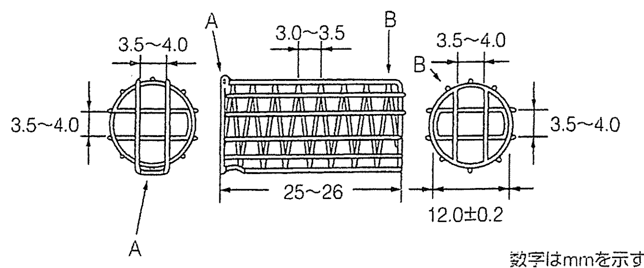

図2 シンカーの仕様例

ればならない．ただし，脈流が生じない送液用ポンプを用いてもよい」とのみ説明されていたが，日局16では，試験条件の一定性確保を明確にするため，さらに「フロースルーセル法による溶出試験では，送液速度と，脈流の有無が規定されなければならない」と追加した．

3 試験液の計量

「操作　回転バスケット法及びパドル法　即放性製剤」の試験液について，「規定された容量は，20～25℃での計量値に相当する」を追加した．この追加部分は，国際調和試験法にはもともとあった記述であるが，試験液はしばしば加熱脱気され，試験液の体積を20℃で計量することは困難であり，多くの場合は，加熱した試験液をメスシリンダーで計量している状況に混乱を起こす可能性があったので，日局15取り込み時に削除した．しかし，その後ICH Q4Bで規制当局による受入の検討の過程で，追加することとした．ただし，この追加部分は，20～25℃で試験液の体積を測定することを意図しているのではなく，例えば，従来通りメスシリンダーで計量し温度換算などの換算を行うような操作でもよい．

点眼剤の不溶性異物検査法

日局15においては，点眼剤の不溶性異物検査法の判定基準は製剤総則の点眼剤各条中に記されていた．しかし，日局16局では以下の判定基準は一般試験法の点眼剤の不溶性異物検査法中の「3．判定」の項に記すこととした．

「澄明で，たやすく検出される不溶性異物を認めない．」

おわりに

既存の製剤試験法については，主立った試験については米国薬局方と欧州薬局方との国際調和はなされたが，各局とも長い歴史をもった試験法であるため，いまだ非調和部分を含むものも少なくない．したがって今後も部分的な改正がなされ，その改正を日局に取り込む改正が続くことが予想される．また，製剤吸入試験関係の新たな試験法の調和が続いており，近々にこれら調和試験法が収載されるものと思われる．さらに，日局16製剤総則には，一般試験法にはまだない，「適切な○○性」と表現された確認すべき製剤特性があげられている．これらの試験については，今後試験法の設定が望まれるところである．

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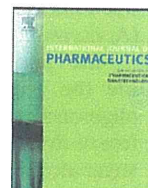

Freeze-drying of proteins with glass-forming oligosaccharide-derived sugar alcohols

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ABSTRACT

Physical properties and protein-stabilizing effects of sugar alcohols in frozen aqueous solutions and freeze-dried solids were studied. Various frozen sugar alcohol solutions showed a glass transition of the maximally freeze-concentrated phase at temperatures (T_g's) that depended largely on the solute molecular weights. Some oligosaccharide-derived sugar alcohols (e.g., maltitol, lactitol, maltotriitol) formed glass-state amorphous cake-structure freeze-dried solids. Microscopic observation of frozen maltitol and lactitol solutions under vacuum (FDM) indicated onset of physical collapse at temperatures (T_c) several degrees higher than their T_g's. Freeze-drying of pentitols (e.g., xylitol) and hexitols (e.g., sorbitol, mannitol) resulted in collapsed or crystallized solids. The glass-forming sugar alcohols prevented activity loss of a model protein (LDH: lactate dehydrogenase) during freeze-drying and subsequent storage at 50 °C. They also protected bovine serum albumin (BSA) from lyophilization-induced secondary structure perturbation. The glass-forming sugar alcohols showed lower susceptibility to Maillard reaction with co-lyophilized L-lysine compared to reducing and non-reducing disaccharides during storage at elevated temperature. Application of the oligosaccharide-derived sugar alcohols as alternative stabilizers in lyophilized protein formulations was discussed.

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1. Introduction

Freeze-drying is a popular method to ensure long-term stability of therapeutic proteins that are not stable enough in the aqueous solutions during distribution and long-term storage (Manning et al., 1989; Nail et al., 2002). Removal of surrounding water molecules by lyophilization significantly reduces gradual chemical and physical degradation of proteins, whereas the dehydration often induces structural perturbation that leads to misfolding and/or aggregation in the re-hydrated solutions (Arakawa et al., 2001). Protecting the protein conformation in freeze-dried formulations through appropriate process control and ingredient optimization is essential to ensure the pharmacological effects, as well as to reduce the risk of product immunogenicity (Hermeling et al., 2004).

Some non-reducing saccharides (e.g., sucrose, trehalose) are popular stabilizers that protect proteins from the chemical and physical degradations in aqueous solutions, during freeze-drying, and in subsequent storage (Arakawa and Timasheff, 1982; Carpenter and Crowe, 1989; Franks, 1992; Wang, 2000). They protect protein conformation in the solids thermodynamically through direct interactions (e.g., hydrogen bonds) that substitute surrounding water molecules and reduce protein chemical degradation kinetically by embedding the protein in a glass-state lower molecular mobility environment. High molecular mobility of the glass-state disaccharide solids, however, induces slow but not negligible chemical degradation of embedded proteins over pharmaceutically relevant timescales. Unexpected exposure of the solids to humid or high-temperature environments often induces physical changes of the dried cakes (e.g., shrinkage) (Breen et al., 2001; Tian et al., 2007).

Application of other excipients that stabilize proteins by themselves and/or in combination with disaccharides would provide further choices to improve the formulation quality (Wang, 2000; Costantino, 2004). Some excipients (e.g., polymers, sodium phosphates) raise the glass transition temperature (T_g) of co-lyophilized disaccharide-based solids, which limited molecular mobility should confer robustness against undesirable storage conditions (Ohtake et al., 2004). Some amino acids and their salts (e.g., L-arginine citrate) form glass-state amorphous solids that protect proteins from inactivation during freeze-drying (Tian et al., 2007; Izutsu et al., 2009). In addition to the structural stabilization, varied physicochemical properties of amino acids would provide some unique effects (e.g., reducing the protein aggregation in aque-

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ous solutions) that are preferable for pharmaceutical formulations (Arakawa et al., 2007).

The purpose of this study was to systematically examine the physical properties and protein-stabilizing effects of oligosaccharide-derived sugar alcohols for their application in freeze-dried formulations. It has been established that various sugar alcohols (e.g., sorbitol, xylitol, lactitol) protect proteins from heat-induced denaturation in aqueous solutions through a thermodynamic mechanism (preferential exclusion) identical to that of saccharides and other polyols (e.g., glycerol) (Arakawa and Timasheff, 1982; Gekko, 1982). Some pentitols and hexitols (e.g., xylitol, sorbitol) protect biological macromolecules (e.g., proteins) and microorganisms from inactivation and/or viability loss during freeze-thawing and during freeze-drying (Tamoto et al., 1961; Carpenter and Crowe, 1988). Varied physical properties (i.e., crystallinity, molecular mobility) have been considered as key factors that determine effects of sugar alcohols to stabilize proteins in frozen solutions and freeze-dried solids (Griebenow and Klibanov, 1995; Carrasquillo et al., 2000; Liao et al., 2002). For example, high propensity to crystallize in the frozen solution (e.g., mannitol) or to collapse during primary drying (e.g., sorbitol, xylitol) makes them inappropriate for main stabilizer in freeze-drying. Some oligosaccharide-derived sugar alcohols (e.g., maltitol, lactitol, maltotriitol) should have greater opportunities to structurally and kinetically stabilize proteins during freeze-drying and subsequent storage. Maltitol and lactitol are popular excipients for oral (tablet) formulations, and are also widely used in food industries as glass-forming additives upon cooling of edible hot-melt compositions (Slade et al., 2006). Information on the physical properties (e.g., thermal transition temperatures) and protein-stabilizing effects (e.g., enzyme activity, protein secondary structure) should be relevant in the application of sugar alcohols to the freeze-dried formulations.

2. Materials and methods

2.1. Materials

All chemicals employed in this study were of analytical grades and were obtained from the following commercial sources: L-lactic dehydrogenase (LDH, rabbit muscle), bovine serum albumin (BSA, essentially fatty acid free), glucose, trehalose dihydrate, sorbitol, and sucrose (Sigma Chemical, St. Louis, MO); maltitol, maltotriitol and maltotetraitol (Hayashibara Biochemical Laboratories, Okayama, Japan); maltose, lactose, mannitol, xylitol, lactitol monohydrate, and other chemicals (Wako Pure Chemical, Osaka, Japan); methanol dehydrate (Kanto Kagaku, Tokyo, Japan). The protein solutions were dialyzed against 50 mM sodium phosphate buffer (pH 7.0), and then centrifuged (1500 g × 5 min) and filtered (0.45 μm PVDF filters, Millipore, Bedford, MA) to remove insoluble aggregates before the freeze-drying study.

2.2. Freeze-drying

A freeze-drier (FreeZone-6; Labconco, Kansas City, MO) was used for lyophilization. Aliquots (0.3 ml) of aqueous solutions in flat-bottom glass vials (13 mm diameter, SVF-3; Nichiden-Rika Glass, Kobe, Japan) were placed on the shelf of the lyophilizer. The shelf was cooled to −40 °C at 0.5 °C/min, and then maintained at this temperature for 2 h before the primary drying process. The frozen solutions were dried under a vacuum (4.0 Pa) while maintaining the shelf temperature at −40 °C for 15 h, −30 °C for 6 h, and 35 °C for 6 h. The shelf was heated at 0.2 °C/min between the thermal steps. The vials were closed with rubber stoppers under a vacuum.

2.3. Thermal analysis

Thermal analysis of frozen solutions and dried solids was performed by using a differential scanning calorimeter (Q-10; TA Instruments, New Castle, DE) and software (Universal Analysis 2000; TA Instruments). Aliquots of aqueous solutions (10 μl) in hermetic aluminum cells were cooled from room temperature to −70 °C at 10 °C/min, and then scanned by heating at 5 °C/min. Freeze-dried solids (1–2 mg) in hermetic aluminum cells were subjected to the thermal analysis from −20 °C at 5 °C/min under a nitrogen gas flow. Cooled-melt saccharide and sugar alcohol solids obtained by a brief period of heating (1 min at 160 °C for maltose monohydrate, xylitol, sorbitol, maltitol, and lactitol monohydrate; at 180 °C for glucose; at 200 °C for sucrose, mannitol, and maltotriitol; and at 220 °C for lactose and trehalose monohydrate) and subsequent rapid cooling (−50 °C) in hermetic aluminum cells were scanned at 5 °C/min to obtain the glass transition temperatures. The glass transition temperatures were determined as the maximum inflection point of the discontinuities in the heat flow curves.

2.4. Freeze-drying microscopy (FDM)

We observed the behavior of frozen aqueous excipient solutions under a vacuum using a freeze-drying microscope system (Lyostat2; Biopharma Technology, Winchester, UK) with an optical microscope (BX51; Olympus, Tokyo). Aqueous solutions (2 μl) sandwiched between cover slips (70 μm apart) were frozen at −40 °C and then maintained at that temperature for 5 min. Each sample was heated under a vacuum (12.9 Pa) at 5 °C/min to a temperature approximately 5 °C below its T_g' as obtained by thermal analysis, and then scanned at an angle speed of 1 °C/min after reaching T_g'. The collapse onset temperature (T_c) of the frozen solution was determined from the first appearance of translucent dots behind the ice sublimation interface ($n = 3$).

2.5. Powder X-ray diffraction (XRD) and residual water measurements

The powder X-ray diffraction patterns were measured at room temperature by using a Rint-Altima diffractometer (Rigaku, Tokyo, Japan) with Cu Kα radiation at 40 kV/40 mA. The samples were scanned in the area of $5° < 2\theta < 35°$ at an angle speed of 5°/min. The lyophilized solids were suspended in dehydrated methanol to obtain residual water by a volumetric Karl-Fischer titrator (AQV-6; Hiranuma Sangyo, Ibaraki, Japan). Residual water contents were shown as ratios (%) to the estimated solid weights in the vials.

2.6. Freeze-drying and activity measurement of LDH

Aqueous solutions (0.5 ml) containing LDH (0.05 mg/ml), excipients (100 mg/ml) and sodium phosphate buffer (50 mM, pH 7.0) were lyophilized in the flat-bottom glass vials. Some freeze-dried solids plugged with rubber stoppers were stored at 50 °C for 7 days in a temperature chamber (Model SH-221, Espec, Osaka, Japan). Pyruvate and NADH were used as substrates to obtain LDH activity from the absorbance reduction at 340 nm (25 °C). Residual enzyme activity was shown as the ratio (%) to that of the solution before freezing ($n = 6$) (Izutsu et al., 1994).

2.7. Fourier-transform infrared (FT-IR) analysis of freeze-dried BSA

A Fourier-transform infrared spectrophotometer (MB-104; Bomen, Quebec, Canada) with a dry gas generator (Balston, Haverhill, MA) and software (PROTA; BioTools, Jupiter, FL and GRAMS/32; Galactic Ind., Salem, NH) was used to obtain mid-infrared spectra of

Table 1
Physical properties of saccharides and sugar alcohols in frozen solutions and freeze-dried solids.

	Excipient				Excipient + BSA + Buffer		
	Frozen solution	Freeze-dried solid		Cooled-melt Solid	Frozen solution	Freeze-dried solid	
	T_g' (°C)	T_g (°C)	Residual water (%, w/w)	T_g (°C)	T_g' (°C)	T_g (°C)	Residual\water (%, w/w)
w/o excipients					n.d.	n.d.	6.3 ± 0.4
Glucose	−42.7 ± 0.5	Collapsed	–	37.3 ± 0.8	−41.4 ± 1.6	41.5 ± 2.0	2.6 ± 0.4
Lactose	−29.1 ± 0.1	90.9 ± 6.6	1.2 ± 0.1	112.0 ± 1.9	−27.8 ± 1.8	105.3 ± 2.2	1.0 ± 0.4
Sucrose	−33.5 ± 0.1	62.0 ± 2.6	1.8 ± 0.9	46.4 ± 0.3	−32.0 ± 0.7	68.2 ± 0.8	1.9 ± 0.0
Maltose monohydrate	−31.1 ± 0.0	86.2 ± 1.1	0.9 ± 0.0	68.8 ± 1.5	−28.8 ± 1.6	95.6 ± 1.2	1.2 ± 0.4
Trehalose dihydrate	−30.6 ± 0.1	80<	1.0 ± 0.2	117.3 ± 0.3	−27.4 ± 0.5	90<	1.2 ± 0.4
Xylitol	−48.5 ± 0.5	Collapsed	–	−21.9 ± 0.2	−45.9 ± 1.2	Collapsed	–
Sorbitol	−45.0 ± 0.4	Collapsed	–	−1.9 ± 0.2	−39.9 ± 0.7	Collapsed	–
Mannitol	Crystallized	Crystallized	–	Crystallized	Crystallized	Partially Crystallized	–
Maltitol	−36.7 ± 0.2	40.6 ± 0.4	1.1 ± 1.1	47.3 ± 0.8	−35.7 ± 0.6	56.3 ± 1.0	1.3 ± 0.4
Lactitol monohydrate	−31.8 ± 0.1	54.9 ± 2.5	0.3 ± 0.5	48.4 ± 3.3	−29.2 ± 1.5	63.3 ± 1.9	1.2 ± 0.2
Maltotriitol	−29.5 ± 0.0	72.8 ± 2.8	0.3 ± 0.2	88.6 ± 0.8	−26.6 ± 0.7	85.3 ± 3.1	1.5 ± 0.2
Maltotetraitol	−24.9 ± 0.2	n.d.	1.2 ± 0.3	–	−24.8 ± 0.3	n.d.	0.9 ± 0.1

Average ± s.d. (n = 3).

BSA in the aqueous solution and freeze-dried solids (Prestrelski et al., 1993; Dong et al., 1995; Izutsu et al., 2004). Spectra of aqueous BSA solutions (10 mg/ml in 50 mM sodium phosphate buffer, pH 7.0) were recorded at 4 cm^{-1} resolution using infrared cells with CaF_2 windows and 6 μm film spacers (256 scans). Spectra of freeze-dried BSA solids were obtained from pressed disks containing the sample (approximately 1 mg BSA) and dried potassium bromide (approx. 250 mg). Area-normalized second-derivative amide I spectra (1600–1715 cm^{-1}, 7-point smoothing) were employed to elucidate the integrity of the protein secondary structure.

2.8. Non-enzymatic color development of freeze-dried solids

Lyophilized solids containing L-lysine (5 mg/ml) and a saccharide or a sugar alcohol (100 mg/ml, 0.3 ml) were stored at 80 °C for 4 days. Changes in the absorbance (280 nm) of the re-hydrated solutions (5-times diluted) were obtained by using a UV–visible spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan).

3. Results

3.1. Physical property of frozen solutions

Most of the frozen aqueous solutions containing a saccharide or a sugar alcohol (100 mg/ml) showed typical thermograms that indicated an amorphous freeze-concentrated phase surrounding ice crystals (Table 1). An increase in the solute molecular weight shifted the glass transition of the maximally freeze-concentrated phase (T_g') to higher temperatures, which trend was consistent with literature (Levine and Slade, 1988). Addition of BSA (10 mg/ml) and sodium phosphate buffer (50 mM, pH 7.0) raised the T_g' of the frozen excipient solutions except for that of maltotetraitol. The frozen mannitol solution showed an exotherm peak that indicated eutectic crystallization at around −23 °C (Cavatur et al., 2002).

Freeze-drying of some disaccharides or oligosaccharide-derived sugar alcohol solutions (trehalose, sucrose, maltitol, lactitol, maltotriitol) resulted in cake-structure solids. Conversely, frozen solutions containing smaller solute molecules (e.g., glucose, sorbitol, xylitol, $T_g' < -40$ °C) collapsed during the process. Addition of BSA prevented glucose from physical collapse during the freeze-drying process. Freeze-drying microscopy indicated dynamic changes of frozen solutions under vacuum (Fig. 1). Heating of a frozen lactitol solution showed ice sublimation from the upper right corner of the image, leaving a structurally ordered dark dried region behind (−30 °C). Further heating induced transparent dots that indicated loss of the local structure (collapse onset temperature, T_c: −27.8 ± 0.3 °C), followed by larger structural damage. Other frozen solutions also showed T_cs (trehalose: −24.3 ± 0.7 °C; maltitol: −31.2 ± 2.1 °C) several degrees higher than their T_g's.

3.2. Characterization of freeze-dried solids

The physical properties of the freeze-dried solids were studied by thermal analysis, powder X-ray diffraction, and residual water measurement. Thermal analysis showed glass transition of some lyophilized oligosaccharide-derived sugar alcohol solids (maltitol, lactitol, maltotriitol) at above room temperature (Table 1) (Shirke et al., 2005). No apparent transition was observed in freeze-dried maltotetraitol solid. Some freeze-dried disaccharides (e.g., lactose, maltose) showed T_gs higher than those of the structurally relating sugar alcohols. Freeze-dried solids containing BSA, buffer components, and disaccharides or oligosaccharide-derived sugar alcohols (maltitol, lactitol, maltotriitol) showed halo powder X-ray diffraction (XRD) patterns typical for non-crystalline solids (Fig. 2). Some peaks in the XRD pattern, as well as the combination of an exotherm peak (51.7 °C) and an exotherm peak (164.1 °C) in the thermogram, indicated partially crystallized mannitol lyophilized with BSA and the buffer salts. The small peaks in the XRD patterns also suggested partial crystallization of glucose and sorbitol during the freeze-drying process and/or during sample preparation for the analysis. The residual water contents of the cake-structure dried solids were less than 2%. The protein lyophilized without the stabilizing excipients showed higher residual water contents.

3.3. Effects on protein stability

The effects of the oligosaccharide-derived sugar alcohols on the protein stability during the freeze-drying process and subsequent storage were studied through the enzyme activity (LDH) and secondary structure (BSA) measurements. The enzyme (0.05 mg/ml) freeze-dried from the sodium phosphate buffer solution (50 mM, pH 7.0) retained approximately 60% of its initial activity (Fig. 3). The disaccharides and oligosaccharide-derived sugar alcohols (100 mg/ml sucrose, trehalose, maltitol, lactitol, maltotriitol) protected LDH from the activity loss during freeze-drying. In contrast, sorbitol and mannitol did not show any apparent effect on the co-lyophilized enzyme activity. The enzyme lyophilized with sorbitol or in the absence of polyols lost most of its activity during storage at 50 °C for 7 days. The disaccharides and oligosaccharide-

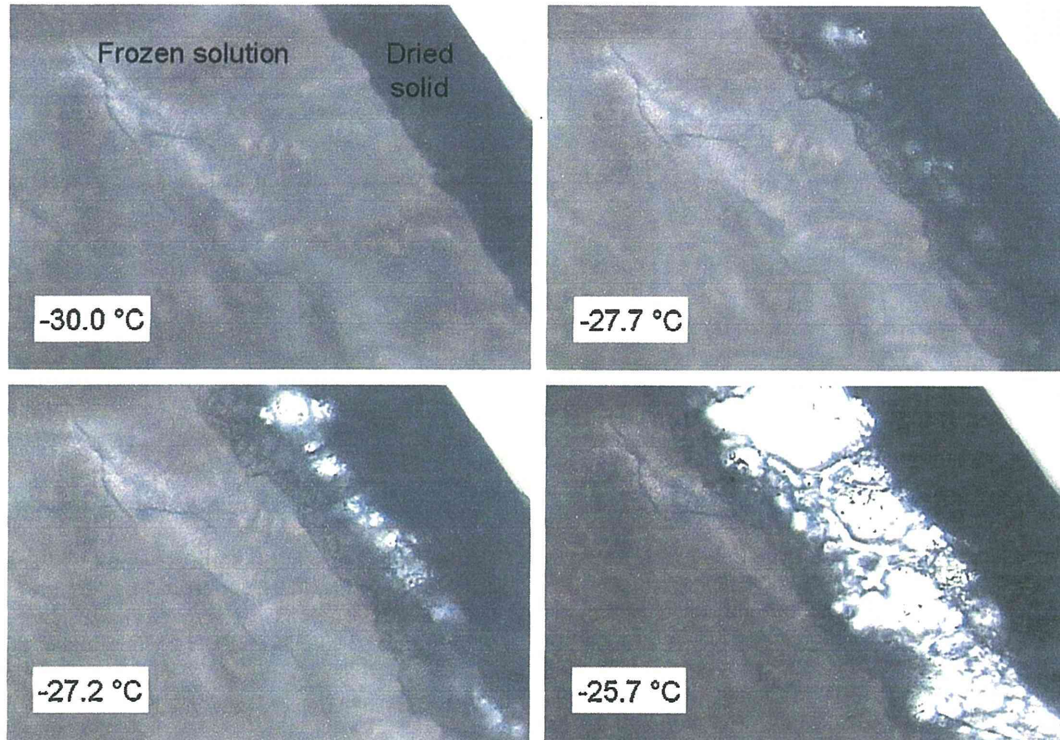

Fig. 1. Freeze-drying microscopy images of a frozen lactitol solution (100 mg/ml) obtained during a heating scan (1 °C/min). The frozen solution (2 μl) in a thin cell was dried under a vacuum (12.9 Pa) from the upper right corner of the figures.

derived sugar alcohols retained the enzyme activity during the high-temperature storage. The freeze-dried maltitol formulation shrunk during the storage near its glass transition temperature. The enzyme lyophilized with mannitol retained its activity to some extent in the largely crystallized solid during the high-temperature storage.

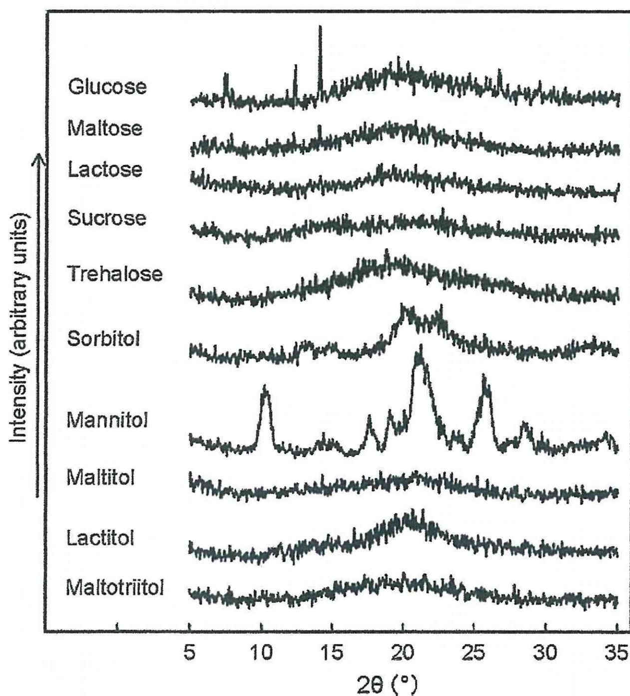

Fig. 2. Powder X-ray diffraction patterns of solids freeze-dried from solutions containing BSA (10 mg/ml), excipient (100 mg/ml) and sodium phosphate buffer (50 mM, pH 7.0).

The effects of the saccharides and sugar alcohols on the secondary structure of freeze-dried BSA were studied (Fig. 4). The area-normalized second-derivative amide I spectra of BSA in the sodium phosphate buffer solution (50 mM, pH 7.0) showed a large band at 1656 cm^{-1} that denoted a predominant α-helix structure in the native conformation (Dong et al., 1995). Lyophilization of the protein from the buffer resulted in a reduction of the α-helix band intensity and broadened the overall spectra, indicating a perturbed secondary structure (Prestrelski et al., 1993). Maltitol and

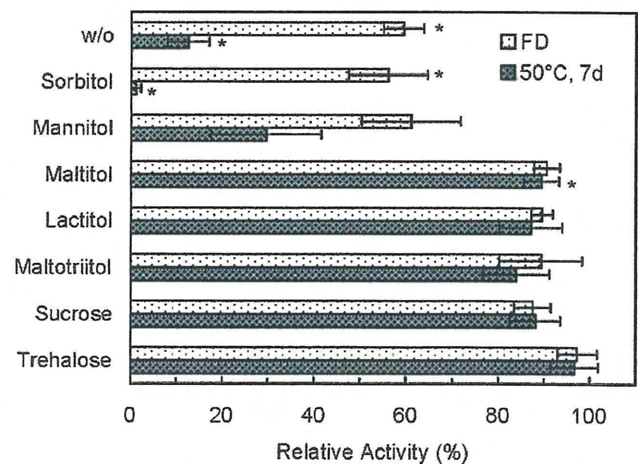

Fig. 3. Effects of excipients on the relative activity of rabbit muscle lactate dehydrogenase after freeze-drying and subsequent storage at 50 °C for 7 days ($n = 3$). Aqueous solutions containing LDH (0.05 mg/ml), excipient (100 mg/ml) and sodium buffer salt (50 mM, pH 7.0) were freeze-dried in glass vials. Asterisks indicate collapsed or shrunk solids.

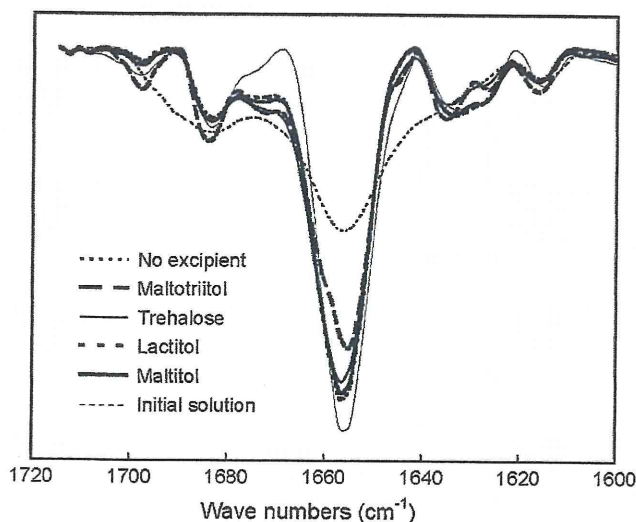

Fig. 4. Area-normalized second-derivative amide I spectra of BSA (10 mg/ml) in a sodium phosphate buffer solution (50 mM, pH 7.0) and in solids freeze-dried with or without co-solutes (100 mg/ml).

lactitol were as effective as trehalose at retaining the conformation of the co-lyophilized protein. The smaller α-helix band of the protein lyophilized with maltotriitol suggested insufficient structure stabilization.

3.4. *Chemical stability in freeze-dried solids*

The possible reactivity of the sugar alcohols with proteins (e.g., Maillard reaction) in the dried solids was studied by using model freeze-dried systems containing the excipients and L-lysine (Fig. 5) (Kawai et al., 2004). The co-lyophilized solids maintained the cake-structure (e.g., trehalose, lactose) or shrunk (other excipients) during the storage at an elevated temperature (80 °C for 4 days). The solids turned brown to varying degrees irrespective of the solid structure. The high-temperature storage of solids containing the reducing saccharides (glucose, maltose, lactose) and L-lysine induced apparent absorbance changes of the re-hydrated solutions (3 < Abs.280, data not shown). The oligosaccharide-derived sugar alcohols (maltitol, lactitol, maltotriitol) showed lower chemical reactivity with co-lyophilized L-lysine

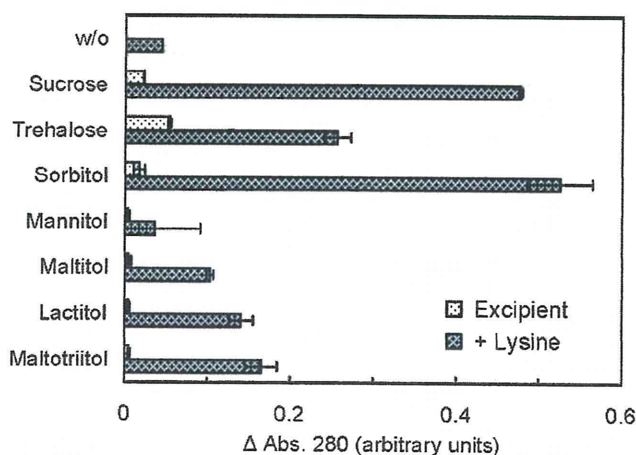

Fig. 5. Effect of storage (80 °C, 3 days) on non-enzymatic color development of solids freeze-dried from solutions containing L-lysine (5 mg/ml) and saccharides or sugar alcohols (100 mg/ml). Changes in the absorbance of re-hydrated solutions were obtained at 280 nm ($n = 3$).

compared to the non-reducing saccharides (sucrose, trehalose). Lower absorbance suggested limited reactivity of the partially crystallized mannitol in the dried solids.

4. Discussion

The results indicated the relevance of some oligosaccharide-derived sugar alcohols as principal stabilizers in the freeze-drying of proteins. An improved understanding the varied physical properties and protein-stabilizing mechanisms in the frozen solutions and freeze-dried solids, in comparison with those of disaccharides, will be indispensable for the rational application of the sugar alcohols.

The thermal transition and collapse onset temperatures (T_g', T_c) of frozen disaccharide-derived sugar alcohol solutions, comparable with those of structurally related saccharides, should allow freeze-drying by ordinary lyophilizers that are designed to cool their shelves down to −40 °C. Decreasing local viscosity of non-crystalline concentrated solute phases above the thermal transition (T_g') induces physical collapse from the drying interface (Pikal and Shah, 1990; Meister and Gieseler, 2009). The collapsed solids are not usually pharmaceutically acceptable because of their inelegant appearance and other changes in their physical properties (e.g., higher residual water, component crystallization) (Costantino et al., 1998). Controlling the shelf temperature to achieve a product slightly below T_g' or T_c (maximum allowable product temperatures) is usually recommended for efficient ice sublimation without collapse, since the ice sublimation speed increases significantly depending on the temperature (approx. 13% at 1 °C interval) (Pikal and Shah, 1990; Nail et al., 2002). Frozen saccharide solutions often show a T_c several degrees higher than the corresponding T_g', which difference depends on various factors, including the component composition and measurement methods (e.g., vacuum pressure, cell structure, type of microscope). Technical difficulties in distinguishing the changes at the collapse onset may partly explain the relatively large difference between the T_g' and T_c in the higher concentration (100 mg/ml) frozen excipient solutions.

The disaccharides and oligosaccharide-derived sugar alcohols formed cake-structure glass-state solids upon freeze-drying. Varied solid densities, degradation products, and residual water contents originating from the hydrated crystals may explain the different T_gs of some excipients prepared by freeze-drying and quench-cooling of the heat-melt. Addition of BSA and the buffer salts raised the transition temperature of the frozen solutions (T_g') and the dried solids (T_g) containing the oligosaccharide-derived sugar alcohols, suggesting their molecular-level mixing in the freeze-dried solid. Possible large molecular mobility during primary (low T_g' of frozen solutions) and/or secondary (low T_g of partially dried solids) drying processes should explain the partial crystallinity of glucose and sorbitol in the solids (Piedmonte et al., 2007).

The retention of the enzyme activity (LDH) and secondary structure (BSA) indicated that the oligosaccharide-derived sugar alcohols protected the proteins against stresses in each step of the freeze-drying process. LDH is a typical enzyme that irreversibly loses its activity by freeze-thawing and freeze-drying-induced subunit dissociation and conformation changes (Jaenicke, 1990; Anchordoquy et al., 2001; Bhatnagar et al., 2008). Various sugar alcohols (e.g., sorbitol, xylitol, maltitol) favor the native conformation of proteins over the unfolded states in the aqueous solutions in the same thermodynamic mechanism with those of saccharides (e.g., preferential exclusion) (Arakawa and Timasheff, 1982; Gekko, 1982; Gekko and Idota, 1989). In addition to the stabilization of aqueous proteins prior to freeze-drying and after re-hydration, some sugar alcohols (e.g., xylitol, sorbitol) is considered to protect proteins from low-temperature-induced conformational changes in frozen solutions through the thermodynamic mechanism (Carpenter and Crowe, 1988; Arakawa et al., 2001). Sta-

bilization of proteins and cell membranes makes sorbitol a popular additive for food cryopreservation (e.g., minced fish meat) (Suzuki, 1981).

Extent of conformation changes by dehydration during secondary drying usually determines the lyophilization-induced protein inactivation (Jiang and Nail, 1998). The oligosaccharide-derived sugar alcohols (e.g., lactitol, maltitol) should substitute water molecules surrounding proteins that are essential to maintain the conformation during the freeze-drying process, as has been reported in oligosaccharides (Carpenter and Crowe, 1989). Insufficient number of water-substituting hydrogen bonds due to steric hindrance may explain the smaller structure-stabilizing effect of maltotriitol compared to maltitol and lactitol. Similar reductions of the structure-stabilizing effects have been reported in some larger oligosaccharides (e.g., maltotriose, maltotetraose, maltopentaose) and polysaccharides (e.g., dextran) (Tanaka et al., 1991; Izutsu et al., 2004). Crystallization during the freeze-drying process and storage deprives some sugar alcohols (e.g., mannitol, sorbitol) of the water-substituting molecular interaction (Izutsu et al., 1993; Cavatur et al., 2002; Piedmonte et al., 2007). Some non-crystallizing pentitols and hexitols (e.g., sorbitol) can provide additional protein-stabilizing water-substituting interactions in the co-lyophilization with some glass-forming or crystallizing excipients (Chang et al., 2005). Crystallization of mannitol in the frozen mixture solutions allows fast lyophilization that results in cake-structure microporous solids and dispersing amorphous regions containing proteins and protein-stabilizing excipients (e.g., sucrose) (Johnson et al., 2002).

The glass-state freeze-dried oligosaccharide-derived sugar alcohol solids should also protect embedded proteins from the chemical and physical degradation during storage. The high T_g and sufficient water-substituting interactions should make lactitol a preferable protein stabilizer over maltitol and maltotriitol for long-term storage of lyophilized solids (Hancock et al., 1995). The lower T_g amorphous solids are prone to faster chemical degradation and physical changes by the larger molecular mobility during storage and occasional exposure to temperatures above their T_g. Our present results also indicate the superior robustness of freeze-dried trehalose against the high-temperature stresses over the other saccharides and sugar alcohols studied. Co-lyophilization with some high T_g excipients (e.g., polymers) or excipients that intensify molecular interactions between stabilizing excipients (e.g., sodium phosphate) should be a potent method to raise the T_g of the amorphous sugar alcohol solids (Miller et al., 1998; Ohtake et al., 2004). The low enzyme activity remaining in the stored mannitol formulation suggested protection of the protein by rubber-state amorphous mannitol moiety dispersed in the physically stable crystalline cake.

In addition to the water-substitution and glass-embedding mechanisms, the oligosaccharide-derived sugar alcohols should protect protein structure in several other ways. They should dilute the non-ice phase in frozen solutions, and thus prevent protein denaturation by various stresses, including excess concentration of unfavorable co-solutes (e.g., inorganic salt), pH change by buffer opponent crystallization, and contact with ice surfaces. The sugar alcohols should also prevent crystallization of co-lyophilized saccharides (e.g., sucrose) during storage (Bhugra et al., 2007). The higher exclusion volume of larger sugar alcohol molecules (e.g., maltotriitol) should help to retain the integrity of the quaternary structure of LDH against the low-temperature-induced subunit dissociation that leads to irreversible structural change (Jaenicke, 1990; Anchordoquy et al., 2001).

The suggested lower susceptibility for the Maillard reaction should be an advantage to applying the oligosaccharide-derived sugar alcohols for freeze-drying of chemically labile proteins. The Maillard reaction, which often appears as non-enzymatic browning, is one of the major pathways of protein chemical degradation that also leads to biological activity loss (Manning et al., 1989; Kawai et al., 2004). The lower hydrolysis rate compared to some oligosaccharides should explain the limited reactivity of the sugar alcohols (Desai et al., 2007). Sucrose tends to be degraded into reactive reducing monosaccharides (glucose, fructose), as well as highly reactive fructofuranosyl cations during storage (Perez Locas and Yaylayan, 2008).

The oligosaccharide-derived sugar alcohols should be potent options in the formulation design as principal stabilizers that alternate disaccharides and/or an additional excipient to optimize the physical properties of the disaccharide-based formulations. Excipients appropriate for a particular therapeutic protein should vary depending on their chemical and physical stability, as well as their intended use. Further information on the safety and long-term protein stability would facilitate application of the oligosaccharide-derived sugar alcohols for freeze-dried protein formulations.

5. Conclusion

Some oligosaccharide-derived sugar alcohols (e.g., maltitol, lactitol, maltotriitol) formed glass-state amorphous cake-structure solids that protect model proteins from secondary structure perturbation (BSA) and activity loss (LDH) during freeze-drying and subsequent storage. Thermal and FDM analysis indicated applicability of ordinary lyophilizer for their freeze-drying without physical collapse during the process. The dried sugar alcohol solids have lower glass transition temperatures than the structurally related oligosaccharides, whereas lower susceptibility to Maillard reaction during storage should be an apparent advantage for particular applications.

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Ammonium ion level in serum affects doxorubicin release from liposomes

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In this study, we measured the release of drug from liposome-encapsulated doxorubicin (DXR) in human and mouse serum. While human serum did not induce DXR-release, mouse serum significantly induced DXR-release in a temperature- and time-dependent manner. Release of DXR was clearly observed in ultrafiltrated mouse serum, indicating that low-molecular substances affect DXR-release. Therefore, the level of Na^+, Cl^-, NH_4^+, and urea nitrogen in each type of serum was measured. Only the concentration of NH_4^+ in mouse serum was significantly higher than that in human serum. Furthermore, addition of ammonium acetate to human serum induced DXR release at the same level observed in mouse serum. These results indicate that the NH_4^+ concentration in serum might greatly affect the release of DXR from liposomes.

1. Introduction

Recently, various liposomal products have been developed and applied to clinical treatment (Coukell and Brogden 1998; Maurer et al. 2001). It is a global requirement that evaluation standards for liposomal products are established to ensure their quality (Burgess et al. 2002). The main purpose of using liposomalization is to stabilize drugs *in vivo* and to control release. For example, the serum half-life of DOXIL®, which is the antitumor agent doxorubicin (DXR) encapsulated in a PEGylated or so-called 'stealth' liposome, is about 90 h (Fujisaka et al. 2006), while that of injected DXR is less than 1 h (Mross et al. 1988). Therefore, drug release (or leakage) is one of the most important formulation properties of liposomal products for quality assessment. *In vitro* drug-release tests for appropriately measuring drug release from liposomes would be very useful for assessing lot-to-lot variability or the release characteristics of liposome products. At present, however, few studies have examined how we should assess *in vitro* drug-release appropriately. From this standpoint, we have studied whether or not an *in vitro* release test, which is related to *in vivo* stability, can be established. It's preferable that such an in vitro drug-release test is based on the *in vivo* release mechanism and correlates with the *in vivo* release profiles. In order to achieve *in vivo* relevance, drug release should be measured under conditions that are as near as possible to the physiological condition. Thus, as a first step, we have investigated the utility of human or mouse serum in the assessment of DXR release from stealth liposome-encapsulated DXR (DXR-SL).

2. Investigations, results and discussion

DXR-SL were incubated with mouse or human serum at various temperatures (37, 45, or 52 °C), and the ratio of DXR release was measured. As a result, mouse serum induced significant DXR release from DXR-SL in a temperature- and time-dependent manner (Fig. 1). In the case of human serum, however, the DXR-release rate was extremely low, even at 52 °C. To our knowledge, it has not been reported that drug release from liposomes differs greatly between human serum and mouse serum.

To elucidate this difference, DXR release from DXR-SL was measured in the filtrate of each serum after ultrafiltration (3 kDa or 10 kDa cut-off). Ultrafiltrated mouse serum induced significant DXR release, although it was slightly lower than that in unfiltered serum (Fig. 2A). In human serum, the DXR-release rate in the filtrate was also slightly lower than that in unfiltered serum. This result indicates that low molecular substances largely affect the release of DXR induced in mouse serum. When DXR-SL was incubated with rat or bovine serum, in addition to human and mouse serum, only mouse serum induced DXR release from DXR-SL (Fig. 2B). Next, we compared the DXR-release rate in four kinds of serum: two kinds of fresh serum collected from CD-1 mice and BALB/c mice (prepared in our laboratory), commercial mouse serum that had been used in the above tests, and human serum. As a result, significant DXR release was observed in only the commercial mouse serum, while the DXR-release rate in fresh mouse serum was equivalent to that in human serum (Fig. 2C). These results indicate the possibility that the low molecular substances affecting drug release are specific to the commercial mouse serum.

Therefore, we measured the concentration of typical low molecular substances in blood, such as Na^+, Cl^-, NH_4^+ and urea nitrogen, in each type of serum. Surprisingly, the NH_4^+ level of the commercial mouse serum was 100-fold higher than that of human serum (Fig. 3A). Likewise, the NH_4^+ level was significantly high in another commercially available mouse serum. On the other hand, the concentration of urea nitrogen in the commercial mouse serum was one-twentieth of that in human serum. The concentration of sodium or chloride was normal in all serum. Next, we examined the effect of NH_4^+ level on DXR release from DXR-SL. It was expected that the pH of the commercial mouse serum would be higher than that of human serum. However, there were no differences in pH between mouse and human serum (data not shown). Thus, we added ammonium

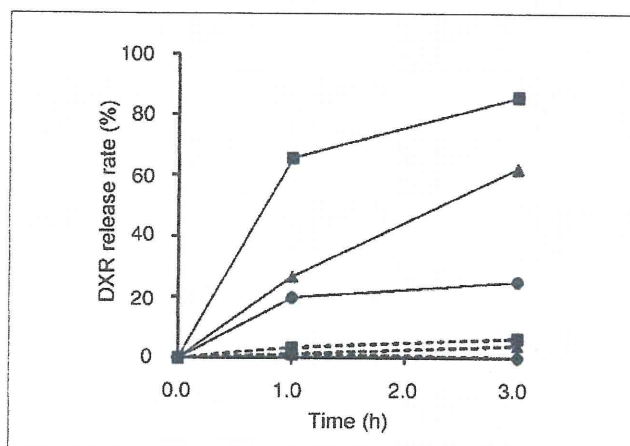

Fig. 1: DXR-release rate in mouse or human serum. DXR-SL (DXR 200 μg/ml) was incubated in human (dashed line) or mouse (solid line) serum (final 90% (v/v)) at 37 °C (circle), 45 °C (triangle), or 52 °C (square) for indicated time

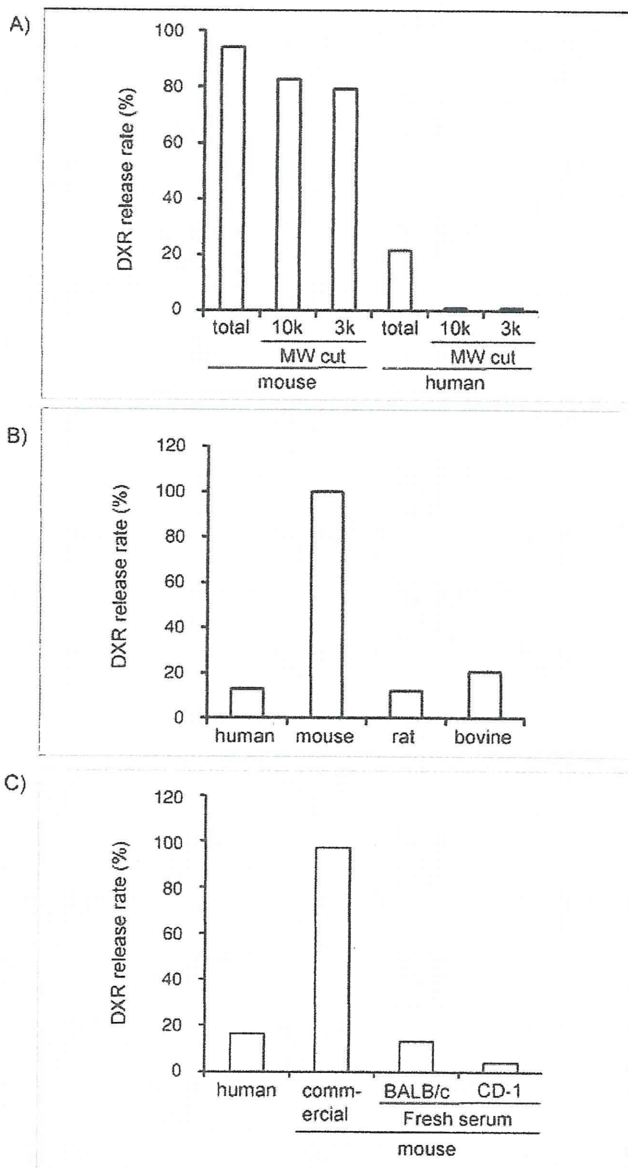

Fig. 2: Effect of difference in serum on DXR-release. A) Effect of ultrafiltrated serum on the DXR-release rate. DXR-SL (DXR 200 μg/ml) was incubated in the filtrate (final 90% (v/v)) for 3 h at 52 °C. DXR-release rate in rat and bovine serum B), and fresh mouse serum collected from BALB/c and CD-1 mice C), in addition to human and mouse serum, were measured after incubation for 3h at 52 °C

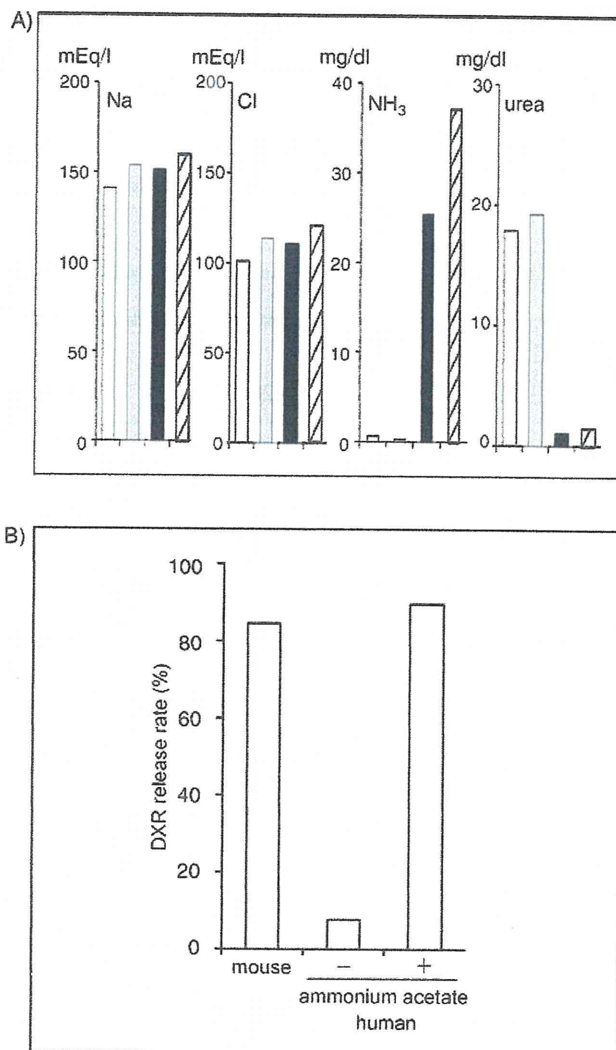

Fig. 3: NH_4^+ level affects DXR release. A) Na^+, Cl^-, NH_4^+ and urea nitrogen in human (white column), fresh mouse serum (gray column), commercial mouse serum (black and shaded column) were measured. B) Ammonium acetate was added to human serum at a final concentration of 1.34 mg/ml which is almost same as the NH_4^+ level in mouse serum. The DXR-release rate in this modified human serum was measured as described in Fig. 1

acetate solution to human serum to the same NH_4^+ level in commercial mouse serum without changing the pH, and measured the DXR-release rate in the adjusted human serum. As in mouse serum, significant DXR release was observed in human serum with ammonium acetate (Fig. 3B). These results suggest that the high NH_4^+ level is one of the causes of the high DXR-release rate in commercial mouse serum.

It is unclear why the NH_4^+ level is markedly increased in commercial mouse serum. Commonly, the blood NH_4^+ level should be measured immediately after blood drawing and centrifugation. Hemolysis and leaving the samples as whole blood at room temperature are causes for elevated test values (Howanitz et al. 1984; Lindner and Bauer 1993). AMP deaminase in red blood cells catalyzes the production of ammonia from protein and amino acids (Nathans et al. 1978). Although we did not investigate the effects in full, we found that, even in human serum, repeating freeze-thaw cycles and long storage tended to increase the DXR-release rate (data not shown). Thus, the high NH_4^+ may be due to a delay in collecting serum after blood drawing, hemolysis, repeating freeze-thaw cycles, or long storage. It is important to stress, however, that the commercial mouse serum used in our examinations is fully compatible with immune assays, such as ELISA or immunostaining, for which it is generally used.

DXR is encapsulated in liposomes by a remote loading method based on the gradient of ammonium sulfate. The mechanism of accumulation is believed to be as follows (Haran et al. 1993). Removal of ammonium sulfate from the extraliposomal medium of liposomes creates an ammonium sulfate gradient $[(NH_4)_2SO_4]_{lip.}>[(NH_4)_2SO_4]_{med.}$ The very high permeability coefficient of neutral NH_3 leads to fast diffusion of NH_3 into the extraliposomal medium. For every NH_3 molecule that leaves the liposome, one proton is left behind, forming a pH gradient across the liposomal membrane. Because DXR is a weakly basic compound (pKa = 8.25), nonionic DXR in the extraliposomal medium diffuses through the lipid bilayer, is protonated and trapped as an ionic form, and accumulates in the intraliposomal aqueous phase by forming a precipitate with sulfate ions. The process can be summarized as an exchange between NH_3 efflux and DXR influx. Therefore, the addition of high concentration ammonium salt to the extraliposomal phase of DXR-SL may induce NH_3 influx into intraliposomes. As a result, the intraliposomal pH may be elevated, and nonionic DXR may diffuse out through the lipid bilayer of liposomes. While the details of the mechanism remain to be elucidated, we speculate that the significant DXR release from DXR-SL in the commercial mouse serum could be caused by high NH_4^+ levels in this way.

Our data revealed that 1) there was almost no DXR release from DXR-SL in human serum, while mouse serum induced significant DXR release; 2) the high NH_4^+ level in mouse serum, especially in commercial mouse serum, is one of the factors leading to the markedly high DXR-release rate; and 3) the concentration of NH_4^+ in the test solution can greatly affect the release of DXR from DXR-SL. Thus, if serum or plasma is used for an *in vitro* drug-release test of liposomal products that are prepared by ammonium sulfate gradient, it will be necessary to control both the lot and the storage period.

3. Experimental

3.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC) and (N-(carbonyl-methoxy polyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG2000) were purchased from Nippon Oil and Fat (Tokyo, Japan). Cholesterol (Chol) was of analytical grade (Wako Pure Chemical, Osaka, Japan). Adriacin® injection 10 (Kyowa Hakko Kirin Co., Ltd.), which is doxorubicin (DXR) injection, was purchased from a general sales agency for drugs in Japan. Mouse, rat serum (Valley Biomedical, Inc., VA), and human serum (Biopredic International, Rennes, France) were obtained from KAC Co., Ltd. (Kyoto, Japan). Another mouse serum was obtained from Cedarlane Laboratories Limited (Ontario, Canada). Bovine serum was purchased from Invitrogen (Carlsbad, CA). Fresh mouse serum collected from CD-1 mice was supplied by Charles River (Kanagawa, Japan). Sepharose CL-4B and Sephadex G-25 prepacked columns, PD-10 Desalting Columns, were purchased from GE Healthcare Japan (Tokyo, Japan).

3.2. Liposome preparation

DXR-SL composed of HSPC/Chol/DSPE-PEG2000 (56.5/38/5.4 molar ratio) was prepared by a modified ethanol injection method (Maitani et al. 2001). DXR was encapsulated into liposomes by remote loading using an ammonium sulfate gradient (Lasic et al. 1992). Briefly, all lipids were dissolved in about 5 ml of ethanol, and the ethanol was removed with a rotary evaporator leaving behind about 1 ml of the ethanol solution. Next, 4 ml of 300 mM ammonium sulfate was added to the ethanol solution. Liposomes formed spontaneously after further evaporation of the residual ethanol. After five freeze-thaw cycles, liposomes were extruded through a series of polycarbonate filters (Nucleopore, CA) with pore sizes ranging from 0.4 to 0.1 μm. The mean diameter of resulting liposomes was determined by dynamic light scattering using a DLS-7000 (Otsuka Electronics Co. Ltd., Osaka, Japan). The diameter of extruded liposomes was in the range of 110 ± 30 nm. Following extrusion, liposomes were ultracentrifuged at 80,000 rpm for 45 min at 4 °C, and suspended in normal saline. The concentration of phospholipid was determined by colorimetric assay using Phopholipids C (Wako Pure Chemical Industries, Ltd., Osaka, Japan). DXR was added to the liposomes at a DXR/liposome ratio of 0.2:1 (w/w), and liposomes were incubated for 1 h at 55 °C. The liposome-encapsulated DXR, DXR-SL, was exchanged by eluting through a PD-10 Desalting Column equilibrated with normal saline.

3.3. Release of doxorubicin

DXR-SL (DXR 200 μg/ml) was incubated in each serum (final 90% (v/v)) for indicated time at 37, 45 or 52 °C. After incubation, samples were passed through a Sepharose CL-4B column equilibrated with normal saline to separate the liposomal DXR from serum protein and free drug. The fraction of liposomal DXR was mixed with an equal volume of hydrochloric acid/isopropanol, and the fluorescent intensity was read at 590 nm (excitation 470 nm). The release rate was calculated from the amount of liposomal-DXR. For ultrafiltration, 4 ml of each serum was ultrafiltrated on centrifugal filter units (NMWL 10k or 3k, AmiconUltra, Millipore Corporate Headquarters, Billerica, MA), and 2 ml filtrate was used for release assay. Ammonium acetate was dissolved in water (134 mg/mL) and added to human serum at a final concentration of 1.34 mg/mL which is almost same as the NH_4^+ level in mouse serum.

3.4. Ion levels in serum

Measurement of Na^+, Cl^-, NH_4^+ and urea nitrogen in each serum was outsourced to the Mitsubishi Chemical Medience Corporation (Tokyo, Japan). Na^+ and Cl^- were measured by electrode method. NH_4^+ and urea nitrogen were measured by indophenol colorimetric method (Fujii-Okuda method) and urease-LEDH method, respectively.

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Effects of Solute Miscibility on the Micro- and Macroscopic Structural Integrity of Freeze-Dried Solids

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ABSTRACT: The purpose of this study was to elucidate the effect of solute miscibility in frozen solutions on their micro- and macroscopic structural integrity during freeze-drying. Thermal analysis of frozen solutions containing poly(vinylpyrrolidone) (PVP) and dextran showed single or multiple thermal transitions (T'_g: glass transition temperature of maximally freeze-concentrated solutes) depending on their composition, which indicated varied miscibility of the concentrated noncrystalline polymers. Freeze-drying of the miscible solute systems (e.g., PVP 10,000 and dextran 1060, single T'_g) induced physical collapse during primary drying above the transition temperatures ($>T'_g$). Phase-separating PVP 29,000 and dextran 35,000 mixtures (two T'_gs) maintained their cylindrical structure following freeze-drying below both of the T'_gs ($<-24°C$). Primary drying of the dextran-rich systems at temperatures between the two T'_gs (-20 to $-14°C$) resulted in microscopically disordered "microcollapsed" cake-structure solids. Freeze-drying microscopy (FDM) analysis of the microcollapsing polymer system showed locally disordered solid region at temperatures between the collapse onset (T_{c1}) and severe structural change (T_{c2}). The rigid dextran-rich matrix phase should allow microscopic structural change of the higher fluidity PVP-rich phase without loss of the macroscopic cake structure at the temperature range. The results indicated the relevance of physical characterization and process control for appropriate freeze-drying of multicomponent formulations. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 99:4710–4719, 2010

Keywords: freeze-drying/lyophilization; formulation; thermal analysis; calorimetry (DSC); amorphous; glass transition

INTRODUCTION

Increased clinical relevance of various parenteral biopharmaceuticals and drug delivery system formulations emphasize the advantage of freeze-drying for ensuring long-term stability due to reduced molecular mobility.[1–4] The freeze-drying, however, exposes the compounds to freezing and dehydration stresses that often damage their higher order structure, which is essential for the biological activity and other pharmaceutical functions. Optimizing the excipient compositions (e.g., stabilizer, pH-adjusting salt, tonicity modifier) and process parameters for the particular active ingredients or delivery system are inevitable to achieve desirable formulation quality and an efficient drying cycle.[5–7] Controlling the shelf temperature and chamber pressure during the primary drying segment for ice sublimation is of particular importance because of its energy-intensive nature and the large effects on the physical (e.g., solid structure, residual water content, component crystallinity) and functional (e.g., protein activity, drug delivery) properties of the formulations.[8–11]

The rationale for the freeze-drying process optimization has been established primarily for low-molecular-weight pharmaceutically active ingredients and mixtures of the APIs with excipients (e.g., antibiotics and tonicity modifier).[8–10,12] Freezing of aqueous solutions concentrates solutes into the nonice-phase until high viscosity of the supercooled solution (70–80%, w/w) kinetically prevents further ice growth. Each solute has a different propensity to crystallize or remain amorphous in the freeze concentrate. A higher product temperature during the primary drying usually allows faster ice sublimation;[12] however, a significant increase in the mobility of hydrated molecules above certain highest allowable product temperatures often alters the structure of solute systems in the process (meltback

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and collapse).[5,12] Primary drying of the crystallizing (e.g., NaCl, mannitol, poly(ethylene glycol) (PEG)) and noncrystallizing (e.g., saccharides) single-solute frozen solutions is performed at product temperatures slightly lower than their eutectic crystal melting temperature (T_{eu}) and collapse temperature (T_c), respectively, to satisfy reasonable ice sublimation speed and to avoid pharmaceutically unacceptable changes (e.g., inelegant appearance, higher residual water, reduced dissolution rate). Recent improvements in freeze-drying microscopy (FDM) have enabled collapse temperature measurements to be carried out in a reasonable operation time.[12–16] The glass transition temperature of maximally freeze-concentrated solutes (T'_g) obtained by thermal analysis is often used as a surrogate of the T_c. Various solute combinations (e.g., oligosaccharides) miscible in a freeze-concentrated nonice-phase show single T'_g, which is necessary for determining the primary drying temperatures.[17]

Setting appropriate freeze-drying process parameters for frozen solutions and/or suspensions containing heterogeneous freeze-concentrated phases is often more challenging because the varied physical properties (e.g., crystallinity, viscosity) of the individual phases have profound impacts on the occurrence of collapse phenomena. Some polymers (e.g., large poly(vinyl pyrrolidone) (PVP) and dextran) that are miscible in their lower concentration aqueous solutions separate into multiple freeze-concentrated phases predominant in one of the polymers, showing different transitions (T'_gs) for the individual phases in the thermal analysis.[18–22] Thermodynamically unfavorable interactions between the polymer molecules that cause aqueous two-layer formation in their higher concentration solutions, as well as the excess concentrations caused by ice growth, induce the multiple freeze-concentrated phases.[19,23,24] The polymer miscibilities also depend on various factors including monomer structure, molecular size, concentration ratio, and cosolute compositions. A variety of polymer combinations, including some proteins and polysaccharides, are considered to be immiscible in their frozen solutions.[25–27] Crystallization of some component solutes also induces the heterogeneous concentrated phases in a frozen solution.[28] Colyophilization of a crystallizing (e.g., glycine, mannitol) and a noncrystallizing (e.g., sucrose) solutes above T'_g of the amorphous phase results in microcollapsed cake-structure solids consisting of a crystalline matrix and a locally disordered amorphous phase that protects embedded proteins from dehydration stress.[28] Various suspension formulations containing particles and/or molecular assemblies (e.g., drug delivery system carrier, microorganisms) should form concentrated medium and particle phases surrounding ice crystals. Inclusion of some solutes into small ice crystal also induces microscopic component and physical state heterogeneity in a frozen aqueous solution.[29]

The purpose of this study was to elucidate the relationship between the miscibility of amorphous solutes in frozen solutions and their structural integrity during primary drying. The individual concentrated solute mixture and their unmixed phases in a frozen solution should possess different viscosities dependent on both composition and temperature. The effects of the varied physical properties on the micro- and macroscopic structural integrity during primary drying remain to be elucidated. Some observations regarding unusual collapse phenomena during lyophilization of microorganism suspensions indicate the requirement for a strategic approach in setting the process parameters based on the physical properties.[30] Varied molecular weights of PVP and dextran were used as model systems that show different miscibilities in frozen solutions. Methods for characterizing the multiphase frozen solutions and their application to formulation and process optimization are discussed herein.

MATERIALS AND METHODS

Materials

Chemicals used in this study were purchased from Wako Pure Chemical Co. (NaSCN and dehydrated methanol, Osaka, Japan), Sigma–Aldrich Chemical Co. (PVP 29,000, PVP 10,000, dextran 35,000, average molecular weights, St. Louis, MO), and Serva Electrophoresis GmbH (dextran 1060, Heidelberg, Germany).

Thermal Analysis

Thermal analysis of frozen solutions was conducted using a differential scanning calorimeter (DSC Q-10, TA Instruments, New Castle, DE) with Universal Analysis 2000 software (TA Instruments). An aliquot (10 μL) of aqueous solution in an aluminum cell was cooled to −70°C at 10°C/min and then scanned at 5°C/min. The T'_g was determined from the maximum inflection point of the discontinuities in the heat flow curves.

Freeze-Drying Microscopy

We observed the behavior of frozen aqueous polymer solutions under vacuum using a freeze-drying microscope system (Lyostat 2, Biopharma Technology Ltd, Winchester, UK) with an optical microscope (Model BX51, Olympus Co., Tokyo, Japan). The sample temperature sensor was calibrated using the melting temperatures of ice, naphthalene crystal, and eutectic NaCl crystal as standards. Aqueous solutions (2 μL) sandwiched between cover slips (70 μm apart) were

frozen at −30°C and then maintained at that temperature for 5 min. Each sample was heated under a vacuum (0.097 Torr) at 5°C/min to a temperature approximately 5°C below its T'_g, and then scanned at 0.5°C/min. The observation field was moved during the scan to follow the ice sublimation front. Collapse onset temperature (T_c, T_{c1}) of the frozen solution was determined from the appearance of translucent dots behind the ice sublimation interface ($n = 3$). The initial temperature of severe collapse growth observed in some phase-separating polymer systems was temporarily termed the second collapse temperature (T_{c2}).

Freeze-Drying

A freeze-drier (Freezone-6, Labconco, Kansas City, MO) equipped with temperature-controlling trays was used for lyophilization. Aqueous solutions (800 μL) containing the solutes in flat-bottomed borosilicate glass vials (13-mm diameter, SVF-3, Nichiden-rika Glass Co., Kobe, Japan) were placed on the freeze-drier shelves at room temperature. The shelves were cooled to −32°C at 0.5°C/min and then maintained at that temperature for 2 h to freeze the aqueous solutions. The shelves were maintained at −32°C for an additional 2 h or heated to different temperatures (−28, −24, −20, −16, or −12°C) at 0.2°C/min and then maintained at the temperatures for 2 h before the vacuum drying. Primary drying of the frozen solutions was performed at varied shelf temperatures by maintaining the chamber pressures slightly (0.1–0.2 Torr) lower than the vapor pressures of ice at the designated shelf temperatures to avoid large temperature drop by rapid ice sublimation. After the primary drying at −32°C (0.120 Torr), −28°C (0.231 Torr), −26°C (0.315 Torr), −24°C (0.390 Torr), −22°C (0.471 Torr), −20°C (0.636 Torr), −18°C (0.771 Torr), −16°C (0.936 Torr), −14°C (1.236 Torr), and −12°C (1.236 Torr) for 20 h, the samples were further dried at these temperatures for an additional 4 h under reduced pressure (0.03 Torr). The shelves were heated to 35°C at 0.2°C/min and then dried at that temperature for 4 h (0.03 Torr) for the secondary drying. The vials were closed with rubber stoppers under vacuum. Thermocouples were immersed in three polymer solutions to record the product temperature profiles during the drying process. The structural integrity of the freeze-dried solids was judged from their volume and surface texture (e.g., roughness, bubbles).

Scanning Electron Microscopy Measurements

Morphological study of a roughly crushed freeze-dried solid surface was performed using scanning electron microscopy (SEM) (VE-7800, Keyence Co., Osaka, Japan). Prior to imaging, mounted samples were sputter-coated with gold. The samples were exposed to a 20-kV acceleration voltage at 10 Pa.

Measurement of Residual Water Content

An AQV-7 volumetric titrator (Hiranuma Sangyo, Ibaraki, Japan) was used to determine the amount of water in the freeze-dried solids suspended in dehydrated methanol. The amount of residual water obtained in three experiments (Karl-Fischer method) was shown as the ratio (%, w/w) to the solid content.

RESULTS

Thermal Analysis of Frozen Solutions

Figure 1 shows the T'_gs of frozen solutions containing various molecular weights of PVP and dextran at different concentration ratios (total 100 mg/mL). The transitions of single-solute frozen solutions were observed at −26.8°C (PVP 10,000), −23.3°C (PVP 29,000), −23.3°C (dextran 1060), and −12.1°C (dextran 35,000). The frozen polymer mixture solutions showed single or double T'_g transitions that indicated

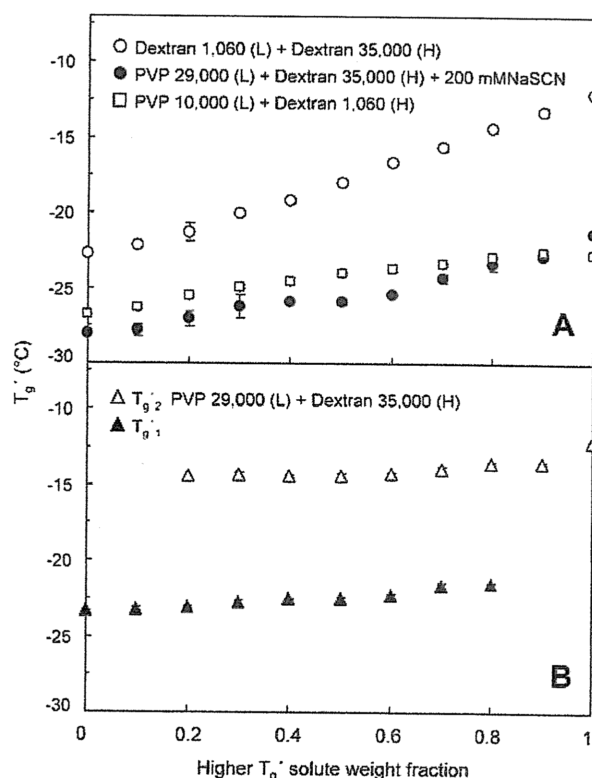

Figure 1. T'_g values of frozen solutions containing PVP and dextran at various concentration ratios (total: 100 mg/mL). The transition temperatures are plotted against the weight concentration ratio of the higher T'_g solute (H) in each combination (average $T'_g \pm$ SD, $n = 3$). The two transitions in a thermal scan are shown as lower (T'_{g1}) and higher (T'_{g2}) temperature transitions.

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different solute miscibility in the freeze-concentrated phases surrounding ice crystals.[21,22] Single transitions that shifted between T'_g of the component solutes (dextran 1060 and PVP 10,000 or dextran 35,000) indicated their freeze-concentration into the same nonice-phase (A). Two transitions at temperatures close to the T'_g of the individual polymers indicated freezing-induced separation of PVP 29,000 and dextran 35,000 into different concentrated phases predominant in one of the polymers (B). The transition temperatures of the PVP-rich (T'_{g1}, lower temperature) and dextran-rich (T'_{g2}, higher temperature) phases rose gradually with the increase in the dextran ratio. The polymer mixture also showed the two T'_gs in freezing from more dilute aqueous solutions (10 mg/mL each, data not shown).[21] Single T'_g transitions observed in some frozen solutions containing predominantly one of the polymers (PVP 29,000 or dextran 35,000, ≥90%, w/w) suggested their miscibility in the freeze-concentrated phase and/or an inapparent transition of the minor phase. Aqueous two-layer formation of the PVP 29,000 and dextran 35,000 mixture solutions was observed at above certain polymer concentrations, dependent on the temperature (120 mg/mL each at room temperature, 80 mg/mL each at −10°C).[21,24,31] Apparent clouding was not observed in the cooling process of the lower concentration polymer mixture solutions (50 mg/mL each) on the lyophilizer shelves. The addition of 200 mM NaSCN merged the two T'_gs, indicating mixing of the polymers in the frozen solutions.[22]

Freeze-Drying Microscopy

We studied the collapse phenomena of the frozen polymer solutions by FDM (Fig. 2).[12–16] Scanning of the frozen solution containing PVP 10,000 and dextran 1060 (50 mg/mL each, A–C) under vacuum showed collapse phenomena typical for the miscible noncrystalline solutes. An advance of ice sublimation on the upper left portion of the image left a dark structurally ordered dried solid layer up to a certain

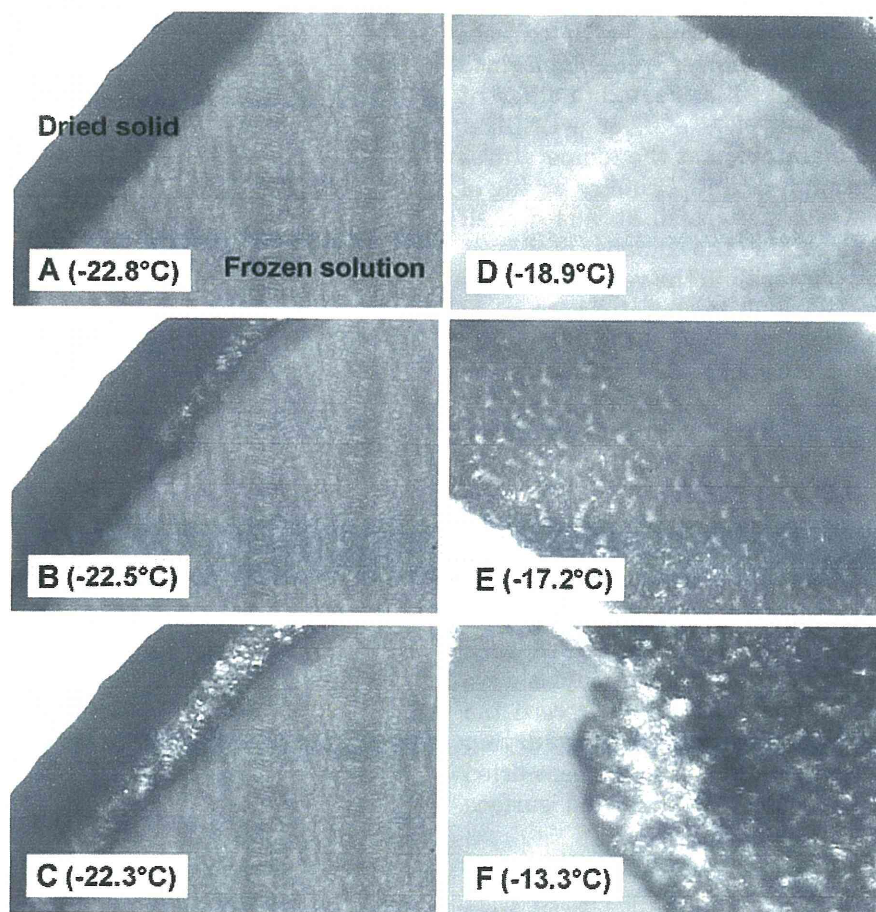

Figure 2. Freeze-drying microscopy images of frozen solutions containing PVP 10,000 and dextran 1060 (**A–C**) and PVP 29,000 and dextran 35,000 (**D–F**) (50 mg/mL each) obtained at different temperatures. The frozen solutions were scanned at 0.5°C/min under reduced pressure (0.097 Torr).