Thiol-Disulfide Interchange in the Tocinoic Acid/Glutathione System During Freezing and Drying

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Abstract

Thiol-disulfide interchange ("disulfide scrambling") is a common mechanism of covalent aggregation for protein drugs. Using tocinoic acid (*cyclo*-S-Cys-Tyr-Ile-Gln-Asn-Cys-(S); TA(ox)) and glutathione (γGlu-Cys-Gly; GSH), our previous work demonstrated that thiol/disulfide interchange is affected by lyophilization in a manner consistent with irreversible and regioselective loss of TA(ox) (Zhang et al., 2009, J Pharm Sci 98/9: 3312–3318). Here, we explore the contributions of stages of the lyophilization cycle to perturbations in thiol/disulfide interchange in the TA/GSH system. TA(ox) and GSH were co-lyophilized from phosphate buffer in the presence or absence of various excipients, then analyzed for TA(ox) and mixed disulfide products by reverse phase high performance liquid chromatography (rp-HPLC). Perturbations were found to occur primarily during freezing, before significant amounts of ice were removed by sublimation. Addition of a lyoprotectant (sucrose), a cryoprotectant (Tween-20) and flash-freezing influenced the product distribution only while ice was still present. Decreasing the redox potential by the addition of oxidized glutathione (GSSG) affected the product distribution differently in lyophilized samples and solution controls, but in neither case led to increased conservation of TA(ox).

Keywords

protein aggregation; peptide; solid state; lyophilization; freeze drying; thiol/disulfide interchange

INTRODUCTION

Therapeutic proteins are often formulated as lyophilized powders to improve stability and preserve efficacy. Nevertheless, proteins can undergo a variety of physical and chemical degradation processes in the solid state that can reduce potency and increase the potential for immunogenic side effects. Aggregation is one of the most frequent types of protein degradation in both solution and solid phases, and can be defined as the formation of covalent and/or noncovalent intermolecular associations among protein molecules. Aggregates have been implicated in life-threatening immunogenic reactions to protein drugs and so must be eliminated during manufacturing, shipping and shelf-storage. One of the most common routes to covalent protein aggregation is thiol-disulfide interchange. The

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reaction mechanism in solution has been well established and involves the pH-dependent generation of the thiolate anion as the rate-determining step, followed by its reaction with the disulfide in an SN2 nucleophilic displacement.[1,2] Whether this mechanism applies to solid forms remains unclear, however, though reducible aggregates have been identified in samples of lyophilized proteins and attributed to thiol-disulfide interchange.[3]

Previous studies in our laboratories explored thiol-disulfide interchange during lyophilization, using tocinoic acid (*cyclo*-S-Cys-Tyr-Ile-Gln-Asn-Cys-(S), TA(ox)) and glutathione (γGlu-Cys-Gly, GSH, reduced) as model peptides.[4] The TA/GSH system is a model for thiol-disulfide interchange in larger proteins, in which native disulfide bonds (i.e., cystine) may react with free thiol groups that are an endogenous part of the protein structure (e.g., Cys side chain) or are formed by partial reduction of disulfides by other means (e.g., β-elimination). Our studies drew on earlier work by Rabenstein and Yeo,[2] who demonstrated that, in solution, TA(ox) rapidly reacts with GSH via thiol-disulfide interchange to produce two singly substituted mixed disulfides, SMD1 ((HS)-Cys-Tyr-Ile-Gln-Asn-Cys-(S)-SG) and SMD2 (GS-(S)-Cys-Tyr-Ile-Gln-Asn-Cys-(SH)) with the disulfide bond at either terminus (Fig. 1).[5] The single mixed disulfides react with GSH to produce the reduced form of TA (TA(red)) and oxidized GSH (GSSG), or with GSSG to form the doubly substituted (or "double mixed") disulfide, DMD (GS-(S)-Cys-Tyr-Ile-Gln-Asn-Cys-(S)-SG) (Fig. 1). The solution reactions were shown to be rapid and reversible, so that equilibrium is established in minutes in neutral solution.[2,5]

Extending these studies to lyophilized solids, we demonstrated that the TA-derived peptides (i.e., TA(ox), TA(red), SMD1, SMD2, and/or DMD; Fig. 1) were present following lyophilization of TA(ox)/GSH solutions from neutral buffer, in keeping with the solution mechanism.[4] However, lyophilization caused a considerable shift in the relative amounts of the five species. During lyophilization, TA(ox) was fully reduced to TA(red). TA(ox) was not detected in samples immediately following lyophilization or after ten days of storage in the solid form, suggesting that the reaction in solids is effectively irreversible. In addition, only one of the single mixed disulfide products (SMD1) was detected in solids, indicating that the reaction occurs preferentially on the less sterically hindered side of the disulfide bond and so is regioselective.[4] These differences in the distribution of TA-derived peptides in the solution and lyophilized solids may be due to temperature-induced changes in the kinetics and/or equilibria of thiol-disulfide interchange reactions during lyophilization, to reduced mobility in the solid samples, or to interactions with the ice surface during freezing. To the extent that the results are applicable to larger proteins, they suggest that native disulfide bonds may be reduced during lyophilization in the presence of free thiol groups. Reduced disulfides may participate in additional reactions during subsequent shelf storage, contributing to instability in the lyophilized product.

While our previous results provide an indication that lyophilization alters the thiol-disulfide product distribution, they are limited in that samples were analyzed only before and after lyophilization. In the work reported here, we identify stages of the lyophilization cycle responsible for process-induced changes and explore the ability of formulation additives (i.e., excipients) and flash freezing to stabilize the native disulfide during lyophilization. The studies are intended to provide mechanistic insight into the factors affecting thiol-disulfide interchange during lyophilization and to evaluate practical strategies for stabilization of native disulfide bonds.

MATERIALS AND METHODS

Chemicals and Reagents

TA(ox), GSH, GSSG, acetonitrile (CHROMASOLV® gradient grade), and sucrose were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). Sodium dihydrogen phosphate (NaH_2PO_4), potassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), and potassium chloride (KCl), all ACS grade, and hydrochloric acid (HCl), phosphoric acid (H_3PO_4, 85%), and Tween-20 (polysorbate 20, enzyme grade) were purchased from Fisher Scientific Co. (Fair Lawn, NJ).

Preparation of Stock Solutions

Stock solutions of TA(ox) (100 μM) and GSH (8 mM) were prepared in phosphate buffer (60 mM K_2HPO_4/KH_2PO_4, pH 7.0, 150 mM KCl). The TA(ox)/GSH reaction mixture for lyophilization, containing 60 mM K_2HPO_4/KH_2PO_4, pH 7.0, 4 mM GSH, 50 μM TA(ox) and 150 mM KCl, was prepared by mixing 100 μL of 8 mM GSH with 100 μL of 100 μM TA(ox) on ice followed by immediate cooling at −80°C for 30 min. Samples were also prepared with the addition of 3% (w/v) sucrose, a common lyoprotectant, or 0.1% (v/v) Tween-20, a pharmaceutically acceptable surfactant and cryoprotectant, to evaluate their ability to preserve the native disulfide. To explore the effects of redox buffer on product distribution, solutions containing both GSSG and GSH were prepared in phosphate buffer (4, 8, or 16 mM GSSG and 8 mM GSH in 60 mM K_2HPO_4/KH_2PO_4, pH 7.0, 150 mM KCl) and mixed with the TA(ox) stock solution. Some excipient-free samples were also subjected to flash freezing in liquid nitrogen at −80°C prior to lyophilization, a process that minimizes the formation of crystalline ice during freezing.

Lyophilization and Sample Analysis

Solid samples were prepared by lyophilization from TA(ox)/GSH reaction mixture using a programmable bench top lyophilizer (VirTis, Gardner, NY) and a fixed lyophilization cycle (Tab. 1). Samples were prefrozen in a −80°C freezer before the lyophilization cycle was initiated (Step 1, Tab. 1). As a control, excipient-free samples (i.e., containing 60 mM K_2 HPO_4/KH_2PO_4, pH 7.0, 4 mM GSH, 50 μM TA(ox), and 150 mM KCl) were wrapped in film, placed in the lyophilizer and exposed to the same temperature cycle as the lyophilized samples. These controls are termed "nonlyophilized," since removal of water by sublimation is prevented by covering the lyophilization vials. In effect, these samples are frozen (and later thawed), but not dried. As additional controls, samples held at room temperature for 5 min and at −80°C for 30 min were also analyzed.

At the end of each lyophilization step, the cycle was stopped and triplicate samples were withdrawn. Fully lyophilized powder samples were reconstituted in 200 μL 0.1 M HCl to quench the reaction. In frozen and liquid samples, the reaction was quenched by adding 4 μL 5 M HCl. The distribution of TA-derived peptides at each lyophilization step was determined by rp-HPLC with u.v. detection at 215 nm, as described previously,[4] using an injection volume of 30 μL. The relative amounts of the five peptides (TA(ox), TA(red), SMD1, SMD2, DMD; Fig. 1) were calculated as a weighted percentage of the total chromatographic peak area at 215 nm. Since synthetic standards were not available for each of the five TA-derived peptides, peak areas were weighted to correct for differences in peptide extinction coefficients at 214 nm (ε_{214}), estimated using the method of Moffatt et al. [6] Estimated ε_{214} values were 25,677 for TA(ox) and TA(red), 31369 for SMD1 and SMD2, and 37061 for DMD. Thus recorded peak areas for SMD1 and SMD2 were multiplied by 0.819 (=25677/31369), and those for DMD by 0.693 (25677/37061). All measurements were performed in triplicate and errors are reported as standard deviations of the mean. Though

this approach is justified for the chemically similar TA-derived peptides, the reported values of "% total peptide" should be regarded as semi-quantitative.

Mass Loss and Water Content

Mass loss was determined by weighing the sample at the beginning and end of each lyophilization step. Water content was analyzed by thermogravimetric analysis (TGA) (Q50, TA Instruments, New Castle, DE). Temperature in the TGA was increased from ambient to 105°C at 10°C/min, followed by an isothermal step at 105°C for 15 min, and then ramping to 150°C at 10°C/min. Mass loss and water content values were determined in triplicate. These measurements confirmed that water removal is complete by the end of 12.5 h, the end of Step 4 of this lyophilization cycle (Tab. 1). No additional mass changes were observed during subsequent steps in lyophilization (not shown).

RESULTS AND DISCUSSION

Effect of Lyophilization on Product Distribution

Changes in the distribution of the five TA-derived peptides (TA(ox), TA(red), SMD1, SMD2, and DMD) during lyophilization are shown in Figure 2. Initially, neutral solution samples contained mostly TA(ox), with smaller amounts of SMD1, SMD2, and DMD (Fig. 2A). TA(red) content was not significantly different from baseline (Fig. 2A). It is reasonable to expect that these initial solution samples of TA/GSH are at equilibrium based on prior literature reports.[2,5] However, the time required to reach equilibrium in frozen or dried samples is not known, so the product distributions of samples taken during lyophilization should not be regarded as equilibrium distributions.

Notable changes in the distribution of TA-derived peptides occurred during freezing (Fig. 2B,C; Steps 1 and 2, Tab. 1). Initial freezing at −80°C for 30 min (Fig. 2B; Step 1, Tab. 1) produced a significant loss in TA(ox) with a corresponding increase in SMD1. TA(red) was present at levels somewhat greater than in the initial solution (Fig. 2A vs. B). Continued freezing at −35°C for 2 h (Step 2, Tab. 1) resulted in additional loss of TA(ox) and further increases in TA(red) (Fig. 2C). Levels of DMD, SMD1, and SMD2 were comparable to those at the end of the preceding step (Fig. 2C vs. B). Throughout freezing, SMD2 and DMD remained at levels similar to those in the initial solution (Fig. 2A vs. B,C).

During primary drying, samples subjected to lyophilization (i.e., in open vials) maintained the TA product distribution produced during freezing, while nonlyophilized samples (i.e., in covered vials) showed increasing reversion to the initial solution distribution (Fig. 2A vs. D,E). Initial primary drying at −35°C for 2 h under 100 mTorr vacuum produced some increases in TA(red) with corresponding loss of SMD1 relative to the previous step. Differences between the lyophilized samples and "nonlyophilized" controls were first observed at this step (Fig. 2D vs. C). Continued primary drying at −5°C for 8 h under 100 mTorr vacuum (Fig. 2E; Step 4, Tab. 1) produced marked differences in the lyophilized and nonlyophilized samples. In lyophilized samples, TA(red) and SMD1 were major products and were present at levels comparable to the previous step (Fig. 2E vs. D), while nonlyophilized samples show some recovery of the parent disulfide, TA(ox) (Fig. 2E).

Continued drying produced few additional changes in either the lyophilized or nonlyophilized samples (Fig. 2F–H; Steps 5–7, Tab. 1). TA(red) and SMD1 remained the major products in lyophilized samples, while the profile in nonlyophilized controls was dominated by TA(ox). At this stage, the nonlyophilized samples were again in solution form, while lyophilized samples were dried powders. Variability in the nonlyophilized samples at the end of the lyophilization cycle may be due to imperfect sealing of the vials

(Fig. 2G), so that some water was lost by evaporation as the samples returned to ambient temperature.

Taken together, the results indicate that changes in the distribution of TA-derived peptides during lyophilization occur primarily during freezing, before water is removed by sublimation (Fig. 2A–C). The results are consistent with: (i) apparent irreversibility of thiol-disulfide interchange in solid samples of TA(ox) and GSH to produce single mixed disulfides and (ii) regioselectivity in the reaction to preferentially form the single mixed disulfide on less hindered side of the bond (SMD1, Fig. 1), as we reported previously.[4] However, these changes may actually reflect physical changes in the sample rather than changes in mechanism. Accordingly, samples were perturbed by flash freezing and by the inclusion of excipients to modify the physical properties of the samples.

Effects of Flash Freezing, Tween-20, and Sucrose

Since changes in the distribution of TA-derived peptides occurred primarily during freezing, it is reasonable to suspect that interactions of the peptides with the ice surface may be involved. For example, adsorption of TA(ox) to the ice surface through hydrophobic or hydrogen-bonding interactions may allow thiol-disulfide interchange to occur as a heterogeneous reaction. Site-specific interactions of TA(ox) with the ice surface (e.g., through hydrogen bonding with the Tyr residue) may promote ring opening on the less hindered side of the TA(ox) disulfide, and thus may partially explain the apparent regioselectivity observed here. Similar site-specific hydrogen-bonding interactions have been implicated in the irreversible adsorption of antifreeze proteins to ice in the tissues of polar fish, an interaction that protects the fish from freezing by limiting ice crystal growth.[7] Interestingly, a sub-class of these naturally occurring antifreeze proteins (Type II) is comprised of disulfide-rich globular proteins.[8] Here, flash freezing and the inclusion of the excipients Tween-20 and sucrose were employed in an attempt to influence any ice-peptide interactions.

Flash freezing limits the time available for ice crystal growth, producing amorphous ice or crystalline forms with smaller crystal size. To accelerate freezing, samples were flash frozen in liquid nitrogen for 30 min prior to initiating the lyophilization cycle. At the end of Step 1, flash frozen samples showed greater retention of TA(ox) and less SMD1 than controls that were not flash frozen (Fig. 3). However, no significant differences in flash frozen and control samples were observed during the rest of the lyophilization cycle (data not shown). Flash frozen samples stored at $-80°C$ for up to 4 days show a product distribution similar to that observed after 30 min (data not shown).

The ability of the cyroprotectant Tween-20 to preserve TA(ox) during freezing was also evaluated. During lyophilization, surfactants such as Tween-20 may compete with the amphiphilic TA-peptides for binding to the ice surface, limiting peptide surface adsorption.[9,10] As with flash freezing, inclusion of Tween-20 resulted in greater retention of TA(ox) and lower levels of SMD1 at the end of Step 1 (Fig. 3), but had no effect on the production distribution during subsequent steps (data not shown).

Carbohydrates such as sucrose and trehalose have been used to retard protein aggregation in amorphous solids, and are thought to act by increasing T_g and limiting mobility in the solid, or by replacing the protein's hydrogen bonds to water to stabilize the native structure.[11–13] To examine the effect of a carbohydrate excipient on thiol-disulfide interchange, 3% (w/w) sucrose was co-lyophilized with the TA(ox)-GSH mixture. Sucrose had no effect on the distribution of TA-derived peptides after 5 min at room temperature (data not shown), but improved retention of TA(ox) during freezing and the initial stage of primary drying (Fig. 4A–C). These differences were not maintained through the later steps of the lyophilization

cycle, however; at Step 4 (Fig. 4D) and subsequent stages, sucrose-containing and control samples showed similar product distributions (data not shown).

The results on the effects of flash freezing and Tween-20 on thiol-disulfide interchange during freezing provide additional support for a role of the ice surface (Fig. 3). As noted above, flash freezing is expected to limit crystal growth while inclusion of the surfactant Tween-20 may interfere with the binding of TA(ox) and/or TA-derived peptides to the ice surface. That both treatments protected TA(ox) during initial freezing (Fig. 3) is consistent with an effect of the ice surface; elimination of these effects during drying (when ice is removed) is an additional indication that they act on the ice-surface. Sucrose may cause similar effects in this system (Fig. 4) by hydrogen bonding to the ice surface and interfering with TA(ox)-ice interactions.

Effect of Redox Buffering

While the data support a role for the ice surface in influencing product distribution, an alternative explanation is that the formation of TA(red) from TA(ox) during freezing is simply the result of a chemically reductive environment (Fig. 2). If this were the case, the addition of oxidized GSSG at constant GSH might be expected to produce changes in redox potential that would protect TA(ox). In solution, increasing levels of GSSG corresponded to increases in DMD and decreases in TA(red) (i.e., increasing R in Fig. 5A, where R = [GSSG]/([GSSG] + [GSH]), a measure of the redox potential). Solution TA(ox) levels decreased only at the highest GSSG content ($R = 0.67$), while SMD1 and SMD2 levels were essentially unaffected by changes in GSSG (Fig. 5A). Similar trends in TA(red) and DMD were observed in lyophilized samples (Fig. 5B). SMD1 showed greater dependence on GSSG in lyophilized solids than in solution, initially increasing with GSSG content to $R = 0.50$ then decreasing at $R = 0.67$. The results are consistent with stoichiometric effects of GSSG and GSH in their reactions with SMD1 and SMD2 to produce TA(red) and DMD in both solution and solid samples (Fig. 1). The lack of an effect on TA(ox) levels is consistent with the constant levels of GSH in these experiments, since GSSG is not involved in the forward or reverse reactions of TA(ox) (k_1, k_{-1}; Fig. 1), and with the relatively slow reverse reaction to form TA(ox) from SMD1 and SMD2 (k_{-1}; Fig. 1). The slow reverse reaction has been reported for TA(ox) and related peptides in solution;[2] our previous studies of the reaction during lyophilization showed this step to be essentially irreversible.[4]

Thus, the addition of oxidized GSSG at constant GSH did not protect TA(ox) and changes in redox potential appear to have little effect in this system. Studies of additional redox buffers in which the species are not also reactants would be interesting and may allow better discrimination of the effects of redox environment and reactant levels.

Implications for Protein Formulation

To the extent that the results obtained with these model peptides are applicable to larger proteins, they have implications for the formulation of lyophilized protein drugs. The results suggest that, in the presence of free thiols, disulfide bonds in proteins may undergo thiol-disulfide interchange during the freezing stage of lyophilization to produce reduced disulfide and single-mixed disulfide products. These process-induced changes can be delayed (i.e., to a later stage in the lyophilization cycle) but not eliminated by the addition of surfactants (e.g., Tween-20), lyoprotectants (e.g., sucrose) or by flash freezing. Similarly, adding a low molecular weight disulfide (e.g., GSSG) alters the product distribution but does not preserve the parent disulfide. Although thiol-disulfide exchange in solution is quenched at low pH, formulating solutions or solids at low enough pH to protonate the thiol (pH <3) is impractical. Thus, limiting free thiol content prior to lyophilization remains as a viable method to prevent the reaction.

Acknowledgments

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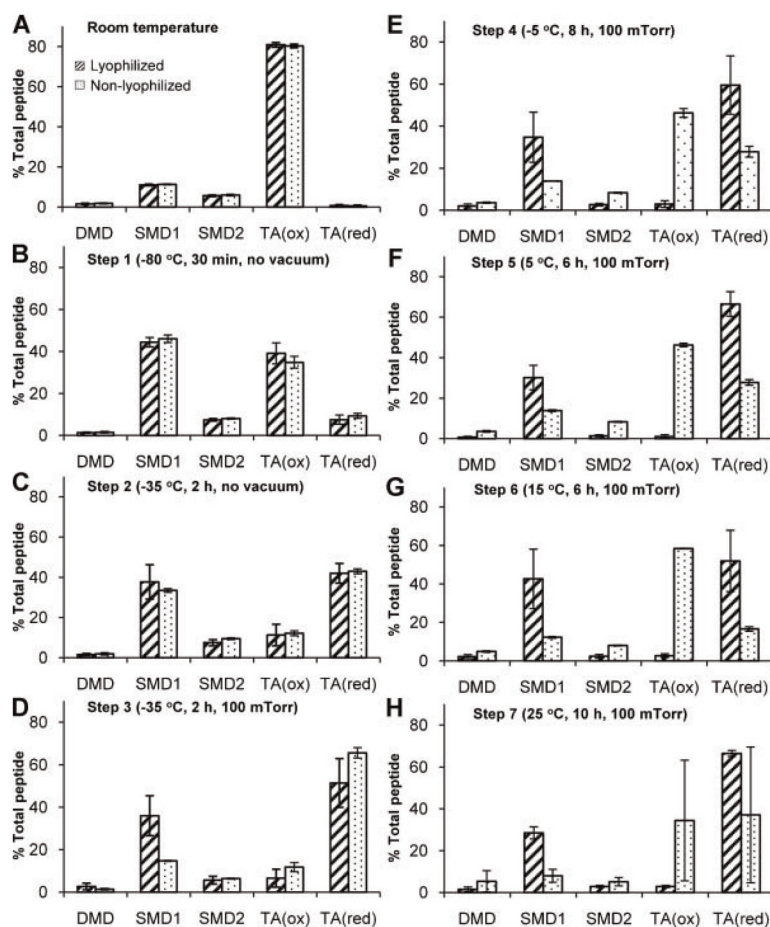
Figure 1.
Thiol-disulfide interchange reactions of tocinoic acid (*cyclo* Cys-Tyr-Ile-Gln-Asn-Cys; TA(ox)) and reduced glutathione (γGlu-Cys-Gly; GSH) in solution. Reaction products are reduced tocinoic acid (TA(red)), two singly substituted mixed disulfides (SMD1, SMD2), the doubly substituted mixed disulfide (DMD), with structures as shown, as well as oxidized glutathione (GSSG).

Figure 2.
Product distribution of the TA(ox)/GSH reaction mixture after the each step of the lyophilization cycle (Tab. 1). Lyophilized samples ("lyophilized," hatched bars) are compared with control samples, which were covered to prevent water removal ("nonlyophilized," stippled bars) but followed the same temperature program. Samples were analyzed after: (A) 5 min in solution at room temperature; (B) 30 min at −80°C without vacuum (Tab. 1, Step 1); (C) an additional 2 h at −35°C without vacuum (Tab. 1, Step 2); (D) an additional 2 h at −35°C with 100 mTorr vacuum (Tab. 1, Step 3); (E) an additional 8 h at −5°C with 100 mTorr vacuum (Tab. 1, Step 4); (F) an additional 6 h at 5°C with 100 mTorr vacuum (Tab. 1, Step 5); (G) an additional 6 h at 15°C with 100 mTorr vacuum (Tab. 1, Step 6); and (H) an additional 10 h at 25°C with 100 mTorr vacuum (Tab. 1, Step 7). Lyophilized samples, $n \geq 6$; nonlyophilized samples, $n \geq 3$; ±SD.
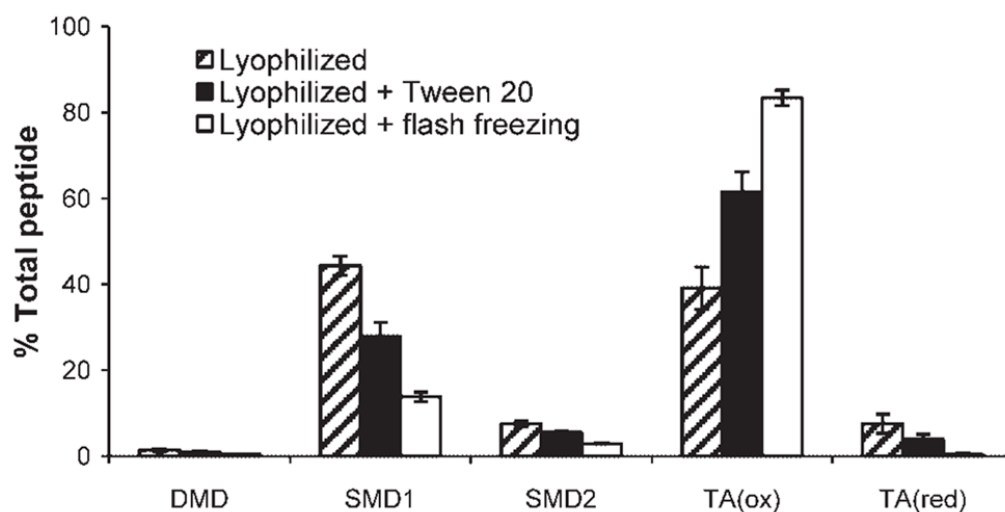
Figure 3.
Effect of flash freezing and Tween-20 on the product distribution of the TA(ox)/GSH reaction mixture at the end of initial freezing at −80°C (Tab. 1, Step 1). Lyophilized samples, $n \geq 6$; lyophilized samples with Tween-20 and flash freezing, $n = 3$; ±SD.
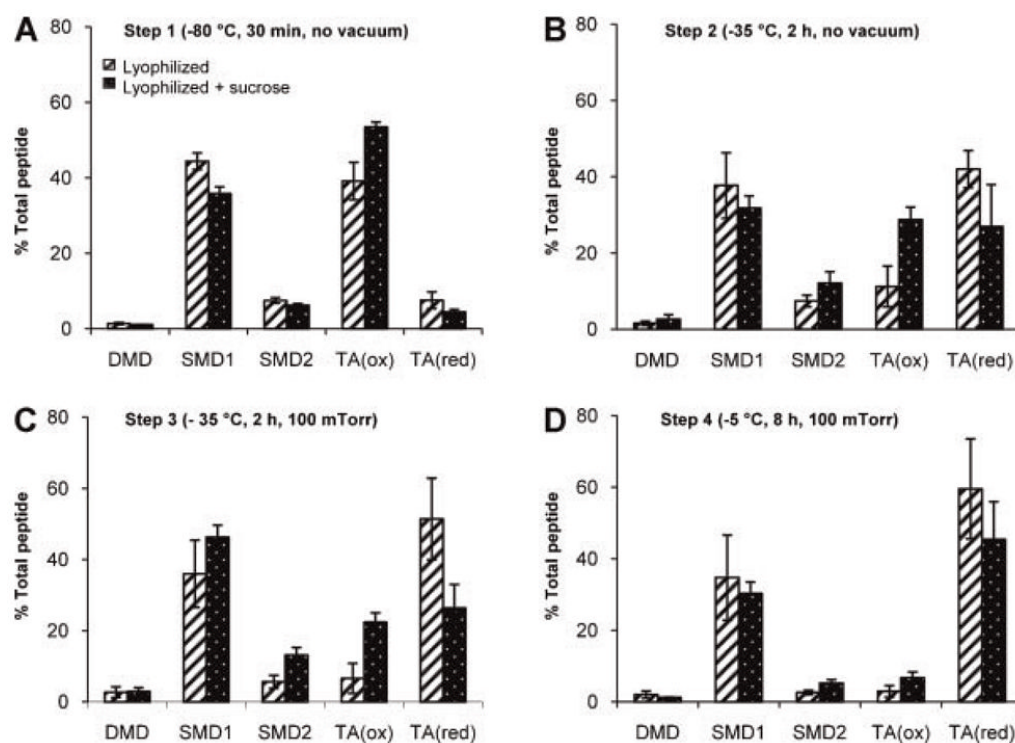
Figure 4.
Effect of sucrose on the product distribution of the TA(ox)/GSH reaction mixture during the first four steps of the lyophilization cycle (Tab. 1). Samples were analyzed after: (A) 30 min at −80°C without vacuum (Tab. 1, Step 1); (B) an additional 2 h at −35°C without vacuum (Tab. 1, Step 2); (C) an additional 2 h at −35°C with 100 mTorr vacuum (Tab. 1, Step 3); and (D) an additional 8 h at −5°C with 100 mTorr vacuum (Tab. 1, Step 4). Lyophilized samples, $n \geq 6$; lyophilized samples with sucrose, $n \geq 3$; ±SD.
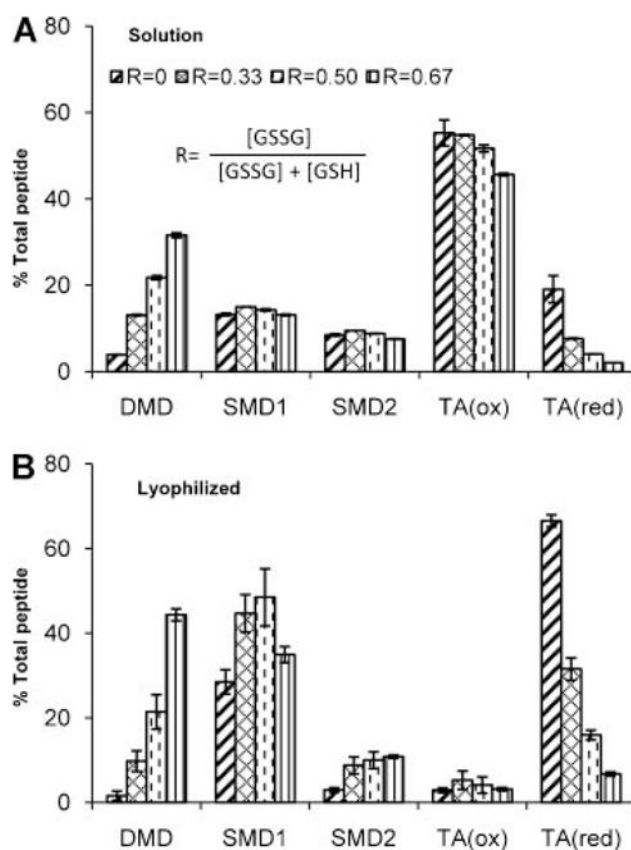
Figure 5.
The effect of GSSG on the product distribution of the TA(ox)/GSH reaction mixture in solution (A) and in fully lyophilized solids (B) (i.e., after Step 7, Tab. 1). The initial solution concentration of GSH was held constant at 8 mM and GSSG was added in varying amounts to produce fractional molar GSSG content (R) of 0, 0.33, 0.50, and 0.67, where $R = [GSSG]/([GSSG] + [GSH])$. $n \geq 3$; ±SD.

Table 1

Lyophilization Cycle for TA/GSH Samples

Step	1	2	3	4	5	6	7
Temperature (°C)	−80	−35	−35	−5	5	15	25
Duration (h)	0.5	2	2	8	6	6	10
Total time (h)	0.5	2.5	4.5	12.5	18.5	24.5	34.5
Vacuum (mTorr)	N/A	N/A	100	100	100	100	60

N/A, not applicable.