ELSEVIER

International Journal of Pharmaceutics 134 (1996) 193–201

international journal of pharmaceutics

Thermal stability of low molecular weight urokinase during heat treatment. III. Effect of salts, sugars and Tween 80

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Received 8 September 1995; revised 26 December 1995; accepted 27 December 1995

Abstract

A turbidimetric assay was used to determine the extent of thermally-induced aggregation in low molecular weight urokinase (LMW-UK). Previous work has shown that, under 60°C heat treatment, LMW-UK denatures and the unfolded protein proceeds to form soluble aggregates. The effects of excipients on the extent of aggregation were examined. Both salts (ammonium sulfate and magnesium chloride) and sugars (sucrose, glucose, trehalose, raffinose) were found to be effective, concentration-dependent inhibitors of aggregation, although excessive salt concentrations did lead to salting out of the protein. Addition of Tween 80, a nonionic detergent, was ineffective. Overall, the effect of these additives on the stability of thermally-stressed LMW-UK can be understood in terms of preferential exclusion of the solute from the surface of the protein. These interactions affect the extent of denaturation, or unfolding, of LMW-UK at 60°C, thereby controlling the degree of aggregation. Purification and incubation experiments indicate that a thermally-unstable subpopulation of LMW-UK exists and is responsible for the majority of the aggregation observed.

Keywords: Urokinase; Thermal stability; Tween 80; Salts; Sugars

1. Introduction

Urinary plasminogen activator (urokinase or UK) is a thrombolytic agent used to dissolve pulmonary emboli and is produced commercially in kidney cell culture. Both high molecular weight

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SSDI 0378-5173(95)04442-0

(HMW-UK, molecular weight ~55 kD) and low molecular weight (LMW-UK, molecular weight ~33 kD) forms of UK have been isolated. Because the LMW-UK produced for pharmaceutical applications (Abbokinase, Abbott Laboratories) is derived from a circulatory organ, there exists a possibility of viral contamination. Consequently, heat treatment to inactivate these viruses is a necessary and required processing step, even if viruses are undetected.

The heat treatment employed for Abbokinase consists of heating the protein in aqueous solution at 60°C for 10 h. This procedure is one which has been approved by the Food and Drug Administration for inactivation of viruses in blood products. Although the process effectively inactivates viral contaminants, the heat treatment also leads to a loss of approximately 20% of the LMW-UK through denaturation and subsequent precipitation. The degraded protein is removed by filtration.

The kinetics of precipitation for LMW-UK during heat treatment exhibits a significant lag time. Our previous work has demonstrated that during the lag time, some LMW-UK denatures, as determined by circular dichroism spectroscopy (Porter et al., 1993). Whether this represents partial denaturation of all the LMW-UK or complete denaturation of a subpopulation had not been determined before this study. As the concentration of denatured protein increases, association of denatured LMW-UK occurs, forming soluble aggregates (Porter et al., 1993). For the purposes of this work, we distinguish between formation of soluble, associated protein (aggregation) and production of insoluble protein (precipitation). Eventually, the soluble aggregates associate to form larger particles that precipitate from solution. During production, this begins to occur after 3–4 h. In a cuvette, with its much smaller volume, precipitation can begin to be seen after approximately 1 h.

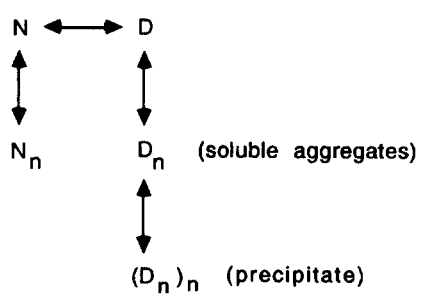
Using a simple turbidimetric assay, we have been able to quantitate the extent of LMW-UK aggregation in the presence of a given excipient (Porter et al., 1993; Vrkljan et al., 1994). The ability of turbidimetric assays to assess the extent of aggregation of proteins quantitatively is well established (European Pharmacopeia, 1980; Martindale et al., 1982; Dathe et al., 1990; Eckhardt et al., 1991; Porter et al., 1993; Volkin et al., 1993; Vrkljan et al., 1994). However, it should be noted that turbidimetric methods do not discriminate between soluble and insoluble aggregates, and do not provide information regarding the size of the aggregates. However, they do allow the effects of additives on the extent of protein aggregation to be assessed (Dathe et al., 1990; Eckhardt et al., 1991; Porter et al., 1993; Volkin et al., 1993; Vrkljan et al., 1994). Although the specific distribution of aggregates in terms of size or weight is not determined, the intensity of the signal was found to be proportional to the signal from conventional light scattering instruments (Porter et al., 1993). Both circular dichroism spectroscopy (Porter et al., 1993) and dynamic light scattering measurements (unpublished results) indicate that most of the protein remains soluble and monomeric, but that a portion of the LMW-UK molecules unfold within minutes and rapidly produce large aggregates, ultimately producing a visible precipitate after 5 or 6 h (Porter et al., 1993).

The optimal solution conditions (pH, ionic strength, protein concentration) for retarding thermally-induced aggregation in LMW-UK in the absence of excipients have been identified (Porter et al., 1993). However, modulation of these extrinsic factors does not eliminate aggregation. Additives have not been used to diminish the loss of Abbokinase during heat treatment due to regulatory concerns. Nevertheless, previous work in our laboratory has shown that polymeric additives (e.g. gelatin, poly(ethylene glycol), heta starch, PVP) do affect the heat-induced aggregation of LMW-UK by stabilizing or destabilizing the native conformation, either through preferential exclusion from or binding to the protein, respectively (Vrkljan et al., 1994). Consequently, monitoring the aggregation of LMW-UK provides a good test case for assessing the effects of excipients on proteins during thermal stress.

For those additives which diminish aggregation, we have shown that stabilization of the native state occurs, meaning less denatured protein at a given temperature (Vrkljan et al., 1994), which, in turn, leads to reduced levels of aggregation (see

Scheme 1). The mechanism of protein stabilization is consistent with the solute exclusion (and binding, for destabilizing solutes) mechanisms, which have been defined in detail by Timasheff, Arakawa and co-workers (Arakawa and Timasheff, 1985; Carpenter and Crowe, 1988; Arakawa et al., 1991; Bhat and Timasheff, 1992; Timasheff, 1992). These researchers have determined experimentally that stabilizers are excluded preferentially from the surface of the protein. This interaction results in an increase in the chemical potential of the protein. Since the degree of exclusion is greater for the unfolded state (which has a larger surface area), there is a larger increase in chemical potential for that form. Thus, the free energy barrier between native and unfolded states is increased, leading to increased structural stability of the native protein.

In this work, we have extended our studies to examine other common classes of stabilizers, including salts, sugars and the nonionic detergent, Tween 80. The heat treatment of LMW-UK provides an ideal system for evaluating the effects on various excipients to retard thermally-induced aggregation in protein pharmaceuticals. Currently, there is a lack of scientific data on which to make rational decisions regarding protein formulations and stabilizers. A broad survey of the effects of commonly employed excipients on a single model protein should be invaluable in providing a framework for comparison. In addition, we describe studies that suggest that there exists a thermally-unstable subpopulation of LMW-UK that is primarily responsible for the aggregation observed in heat-treated LMW-UK.



Scheme 1. General mechanism for thermally-induced aggregation of LMW-UK

2. Materials and methods

2.1. Materials

Purified LMW-UK (pLMW-UK) (lots 20669-pm-159) was obtained from Dr. Jack Henkin at Abbott Laboratories. All other LMW-UK was 'A' fraction quality, where 'A' fraction refers to the main fraction of Abbokinase from the final purification column, but prior to heat treatment. The stock solutions had activities of approximately 150 000–200 000 U/ml and had a protein concentration of 1.3–1.9 mg/ml. They also contained 2.0% NaCl (w/v).

The buffers used were made from stock solutions of 0.2 M sodium monobasic phosphate (Mallinkrodt, lot 7892 KDPV) and sodium dibasic phosphate heptahydrate (Mallinkrodt, lot 7914 KEKC). Mixtures of these two solutions were prepared in order to achieve the proper pH following the method of Gomori (1955).

Tween 80, also known as Polysorbate 80, was obtained from ICI Americas (lot 15635L). Stock solutions of 0.1%, 0.01% and 0.001% Tween 80 in water were prepared by serial dilution. Ammonium sulfate, magnesium chloride, sucrose, glucose, fructose, raffinose and trehalose were of analytical grade and obtained from Mallinkrodt. They were used without further purification.

2.2. Equipment

Both a Cary 219 and a Beckman DU-64 UV-visible spectrophotometer were used for all spectroscopic experiments described in this report. Each was equipped with a Lauda RM-6 circulating water bath in order to maintain a constant sample temperature of 60°C. The cells used were obtained from Hellma, having a pathlength of 10 mm and a sample volume of 1.0–1.5 ml.

2.3. Methods

The spectrophotometer and water bath were allowed to equilibrate for a minimum of 1 h. As described previously, the aggregation of LMW-UK can be monitored by following apparent absorption (i.e. optical density) increases (Porter et al., 1993). In order to maximize sensitivity, the absorbance was measured at 325 nm. There was minimal interference from absorbance changes in the near ultraviolet and monitoring at 325 nm maximized the sensitivity of the measurements. The time course for the reaction was monitored for periods of up to 1 h, beginning with insertion of the sample into the thermostated cell holder. Previously, we demonstrated that the apparent absorption intensity at 30 min correlated with the amount of aggregation, so this was taken as an indicator of turbidity (Porter et al., 1991; Vrkljan et al., 1994). It should be noted that the rate of aggregation observed between 5 and 15 minutes also correlated with the extent of turbidity (Porter et al., 1993). For a typical sample, the sample volume was 1.2 ml and the activity of LMW-UK was 38 500 U/ml (~ 0.33 mg/ml).

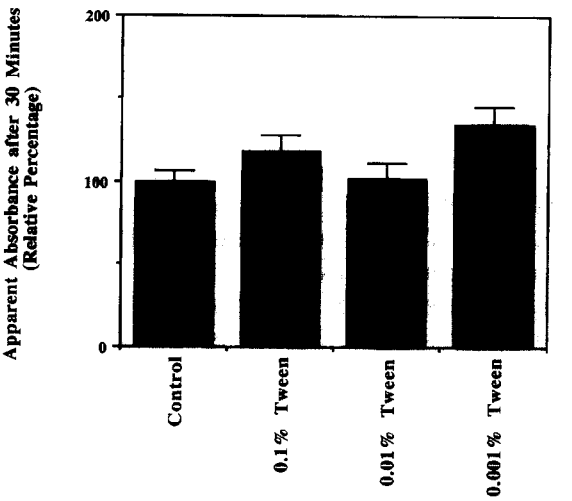


Fig. 1. Extent of aggregation in samples containing Tween 80 as determined by the apparent absorbance at 325 nm after 30 min of heat treatment. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38 500 U/ml and Tween concentrations are in w/v percentages.

3. Results and discussion

3.1. Effects of Tween 80 on the thermal stability of LMW-UK.

A common excipient found in pharmaceutical formulations is Tween 80 (also known as Polysorbate 80), a nonionic detergent (Wang and Hanson, 1988). It is widely reported to be effective at diminishing adsorption to surfaces and to prevent protein-protein interactions at room temperature (Wang and Hanson, 1989; Levine et al., 1991; Patel et al., 1993; Wang et al., 1995; Son and Kwon, 1995). However, its ability to affect the thermal stability of proteins is not well established, although Katakam et al. (1995) have shown that Tween 80 and other nonionic detergents lower the T_m of human growth hormone significantly. Therefore, Tween 80 was examined for its ability to increase the thermal stability of LMW-UK at concentrations both above and be-low its critical micelle concentration (0.008%, cf. Mukerjee and Mysels, 1971), Tween 80 did not diminish the thermal aggregation of LMW-UK (see Fig. 1). In fact, there may be some increase in aggregation in the presence of Tween 80. Most likely, this is due to increased detergent-induced unfolding of LMW-UK, leading to more facile aggregate formation. Similar findings have been reported (Hageman et al., 1993) for the thermally-induced aggregation of bovine growth hormone (bGH). One report on the effects of porcine growth hormone (pGH) appears to contradict this trend (Charman et al., 1993). However, not enough experimental detail was available to determine whether direct comparisons could be drawn.

The concentrations of Tween 80 used in this study lie both above and below the critical micelle concentration. In all cases, the detergent has no effect or is destabilizing. Most likely, this is because a few detergent molecules are binding to the protein at specific sites, rather than the detergent affecting protein interactions with interfaces. This binding then destabilizes the protein to unfolding (leading to increased aggregation) and excess Tween has little or no effect. Specific binding of nonionic detergents has been observed with human growth hormone (Bam et al., 1995).

These results illustrate the danger of extrapolating the stabilizing effects of an additive from one system or stress to another. Many materials may stabilize against one stress but be ineffective against another. In this case, the stress is certainly thermal, because at 25°C, LMW-UK exhibits less than 1% of the aggregation that occurs at 60°C.

3.2. Effect of salts on the thermal stability of LMW-UK

High concentrations of certain salts, such as ammonium sulfate, can stabilize proteins with respect to thermal stresses (Arakawa and Timasheff, 1982a,b, 1984; Ahmad and Bigelow, 1986; Danilenko et al., 1986; Arakawa et al., 1990a,b). Increasing amounts of ammonium sulfate do lead to decreased aggregation of heat-treated LMW-UK (Fig. 2). Beyond approximately 2.5% (w/v, 0.19 M), little further stabilization of LMW-UK occured. At concentrations higher than 22.5% (1.7 M), the protein begins to salt out and precipitate. Salting out and stabilization of a native protein are consistent descriptions of preferential exclusion of the solute (Arakawa et al., 1990a,b).

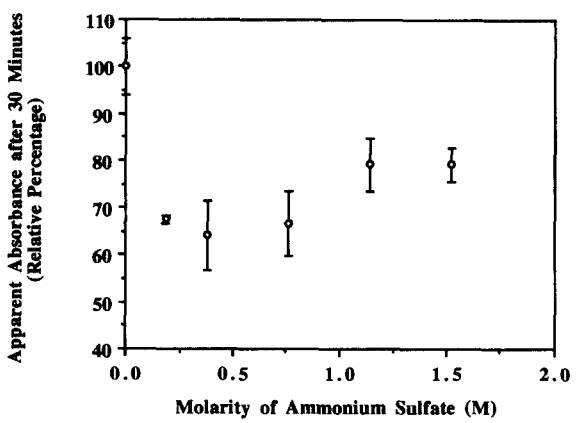


Fig. 2. Extent of aggregation in samples containing ammonium sulfate as determined by apparent absorbance at 325 nm after 30 min of heat treatment. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38 500 U/ml. A 0.75 M solution of ammonium sulfate correlates to a 10% (w/v) solution.

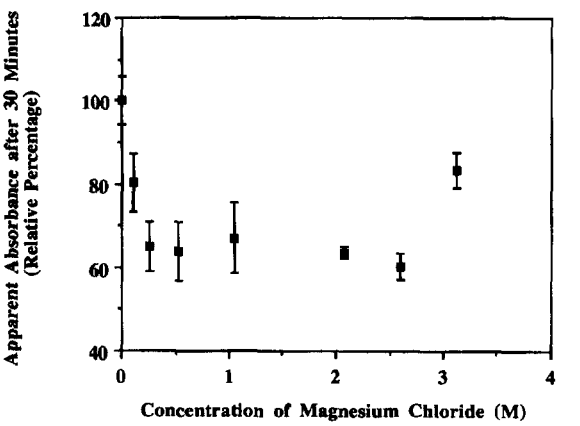


Fig. 3. Extent of aggregation in samples containing magnesium chloride as determined by apparent absorbance at 325 nm after 30 min of heat treatment. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38 500 U/ml. A 1.03 M solution of magnesium chloride correlates to a 10% (w/v) solution.

Magnesium chloride also has been shown to increase protein stability (Arakawa and Timasheff, 1982a, Arakawa and Timasheff, 1994; Arakawa et al., 1990a,b). Even at concentrations as low as 1% (w/v, 0.103 M), $MgCl_2$ was found to significantly stabilize heat-treated LMW-UK (Fig. 3). Maximal stability appears to occur at concentrations of 2.5% and above (> 0.26 M), and is relatively constant up to concentrations as high as 2.6 M. Arakawa and Timasheff have found that $MgCl_2$, unlike ammonium sulfate and other salts, did not always stabilize proteins (Arakawa et al., 1990b). The effect of $MgCl_2$ is more dependent on the pH and the nature of the protein (Arakawa and Timasheff, 1982a,b, 1984), presumably because, at high concentration, the magnesium ions can preferentially bind to the protein, instead of being excluded. However, it appears this is not the case for LMW-UK, as $MgCl_2$ is quite efficient at diminishing heat-induced aggregation. This description of the behavior is further supported by the fact that no correlation has been observed between surface tension effects and stabilization by divalent salts (Arakawa and Timasheff, 1984), in contrast to the behavior of sodium salts and sugars (see below). In each case, it appears that the effect on protein stability is reached rapidly

and plateaus until the concentration where the protein is precipiated through 'salting out'.

3.3. Effect of sugars on the thermal stability of LMW-UK

Simple saccharides are well known to stabilize proteins during stresses such as freezing and drying (Carpenter et al., 1994). Also, it has been shown that the T_m values (the temperature at which one half of the protein is unfolded) for many proteins increase through preferential stabilization of the native state (Lee and Timasheff, 1981; Arakawa and Timasheff, 1985, 1989; Carpenter and Crowe, 1988; Arakawa et al., 1991; Bhat and Timasheff, 1992; Timasheff, 1992).

A number of sugars were investigated for their ability to decrease aggregation of heat-treated LMW-UK. Initial studies employed the disaccharide sucrose, since it is well known as a stabilizer, and its component monosaccharides, glucose and fructose (Arakawa et al., 1991). Fig. 4 shows that on a molar basis, sucrose is more efficient at diminishing heat-induced aggregation than glucose, with increasing stability obtained even at concentrations up to 1.5 M, although they are approximately equal on a monosaccharide basis. Interestingly, fructose causes precipitation of LMW-UK, even at room temperature and relatively low concentrations (5% or 278 mM). There are no data in the literature describing preferential solute interactions with fructose, and no clear structural basis exists for why it acts as a precipitant. However, it is known that stabilization through preferential exclusion does decrease solubility (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982b, 1985; Carpenter and Crowe, 1988; Arakawa et al., 1990a,b, 1991; Bhat and Timasheff, 1992; Timasheff, 1992).

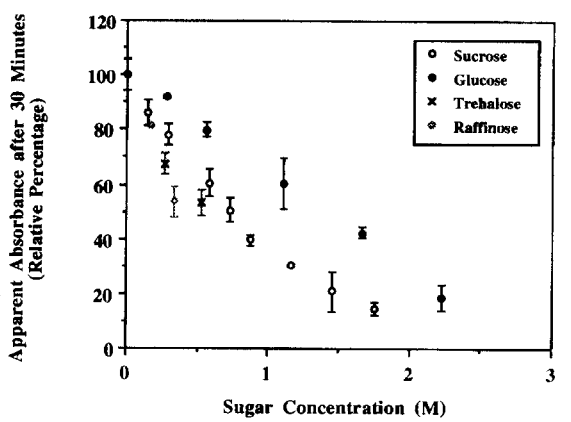


Fig. 4. Extent of aggregation in samples containing sucrose, raffinose, trehalose, and glucose as determined by apparent absorbance at 325 nm after 30 min of heat treatment. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38 500 U/ml.

Lee and Timasheff (1981) have shown that sucrose is highly efficient at stabilizing proteins through preferential exclusion of the solute. Similar studies were reported for glucose (Arakawa and Timasheff, 1982b). Our results are consistent with these earlier studies. Both sugars significantly stabilize LMW-UK with respect to thermal denaturation, leading to less unfolding at 60°C and, therefore, lower levels of aggregation. However, glucose, being a reducing sugar, could produce significant levels of chemically modified protein through the Maillard reaction, so its use as a thermal protectant is limited.

Two other sugars, trehalose and raffinose, were examined for their stabilizing effects (Fig. 4). These oligosaccharides were found to be similar in their stabilization of LMW-UK as sucrose (on a molar basis). No significant difference was observed between trehalose, sucrose and raffinose, suggesting that contrary to some work (Calaco et al., 1994), there is no special stabilization bestowed by trehalose, at least in protecting against thermal denaturation.

Overall, it appears that most oligosaccharides are preferentially excluded from LMW-UK which stabilizes the native structure, and reduce aggregation, following the mechanisms outlined by Timasheff and co-workers (Arakawa and Timasheff, 1985; Carpenter and Crowe, 1988; Arakawa et al., 1991; Bhat and Timasheff, 1992; Timasheff, 1992). The predominant factor for sugars is thought to be through a positive increment in the surface tension, although the nature of the surface of the protein and the excluded volume of the sugar are sure to play a role as well.

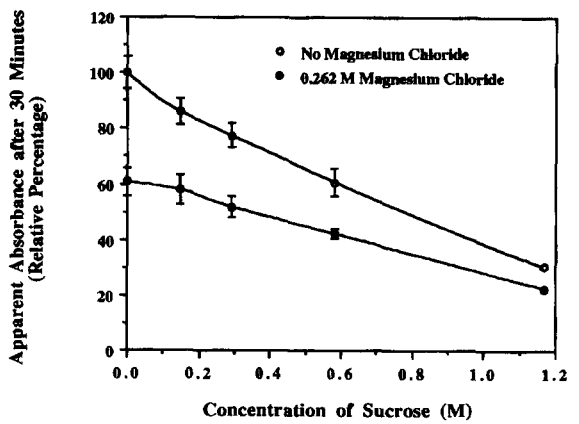


Fig. 5. Extent of aggregation in samples containing sucrose and 0.262 M magnesium chloride as determined by apparent absorbance at 325 nm after 30 min of heat treatment. The error bars represent ± 1 standard deviation. The LMW-UK concentration is 38 500 U/ml.

3.4. Effect of combinations of sucrose and magnesium chloride

One interesting possibility with preferential exclusion mechanisms is that different classes of stabilizers might work in concert to achieve increased stability. This has been demonstrated for glycerol and PEG 4000 in the stabilization of LMW-UK (Vrkljan et al., 1994). However, neither additive was used at its optimal concentration.

Using 0.26 M $MgCl_2$, where the stabilization for the salt is maximal, various amounts of sucrose were added to determine if additional protection against thermally-induced aggregation could be achieved. Addition of sucrose did further protect from thermally-induced aggregation (Fig. 5), especially at high concentrations (> 20% w/v). The two curves appear to be converging, suggesting there is some limit to the maximal amount of stabilization which can be achieved.

This experiment also indicates that maximal stability conferred by one solute may not represent the maximal stabilization possible for the protein. Therefore, use of combinations of additives to achieve increased stability is justified. In the case of magnesium chloride, a minimal amount is required to significantly decrease the amount of sucrose required. It should be noted that the effect is not synergistic. Also, the amounts required are hypertonic. Therefore, use of excipient mixtures may be limited by biological constraints.

3.5. Effect of purification on LMW-UK thermal stability

Highly purified LMW-UK (pLMW-UK) refers to material that has undergone additional chromatographic purification using a benzamidine affinity column. In comparison to commercial LMW-UK, which was used in all other studies, pLMW-UK displays enhanced thermal stability with respect to aggregation during heat treatment. The extent of aggregation for pLMW-UK is decreased by almost ten-fold relative to LMW-UK itself. These results suggest that additional chromatographic purification removes a LMW-UK component which is thermally unstable relative to the rest of the material present. Presumably, it is this component which produces most of the precipitated protein found after 10 h of heating. Polyacrylamide gel electrophoresis does indicate the presence of more than one protein component (results not shown), mostly from N-terminal proteolytic cleavage. Therefore, the aggregation of heat-treated LMW-UK seems to be primarily due to the presence of an unstable component. This also presents the possibility that the partial unfolding described previously may be more complete unfolding of a subpopulation of LMW-UK molecules.

Another important facet of the purification studies is the nature of the initial thermal denaturation step in the thermally-induced aggregation of LMW-UK. According to the mechanism defined in our earlier work (see Scheme 1), either LMW-UK partially unfolds or a less stable subpopulation of LMW-UK completely unfolds, triggering subsequent aggregation of the unfolded state. Incubation of LMW-UK for 2 h, followed by centrifugation, produced a sample which showed similarly diminished susceptibility to aggregation as pLMW-UK (results not shown). Together, these data suggest that, in fact, it is a subpopulation of LMW-UK which denatures completely and leads to subsequent aggregation, rather than some of a single population of LMW-UK undergoing partial denaturation. Most likely, some native LMW-UK becomes included in the formation of these soluble aggregates, but the initial denaturation appears

to involve the less stable fraction almost exclusively.

Apparently, the addition of certain additives (and to a lesser degree, change in ionic strength or pH) raises the thermal melting temperature (T_m) of this subpopulation enough to markedly affect the extent of aggregation and precipitation by preferential interactions with the protein. Preliminary studies indicate that the rest of the LMW-UK molecules must be incubated to at least 75°C in order to achieve the same level of aggregation. Note that this does not mean the T_m for the less stable subpopulation is at 60°C. Presumably, all that is required for structural destabilization is onset of denaturation, that is, for there to be some critical amount of denatured protein present at 60°C.

4. Conclusion

We have found that LMW-UK can be stabilized by the addition of salts and sugars, consistent with a mechanism of stabilizing against unfolding. Such stabilization most likely derives from preferential exclusion of the solute from the protein. However, certain materials (e.g. ammonium sulfate or fructose), if added at sufficiently high concentration will cause precipitation of the protein through salting out. Magnesium chloride was found to be highly effective, as were glucose, sucrose, raffinose and trehalose. On a molar basis, glucose was the least effective of the stabilizing sugars.

Also, we have demonstrated that further purification of LMW-UK appears to remove a thermally sensitive fraction of LMW-UK which probably leads to the majority of the protein lost during heat treatment. Currently, it is unknown as to the extent of protein recovery during benzamidine affinity chromatography. However, if the loss is small, it promises to provide a much more stable LMW-UK mixture. In any case, it is strongly suggestive that heat-induced aggregation is the direct result of a denaturation of a thermally unstable subpopulation of LMW-UK.

Acknowledgements

The financial support of the Thrombolytics Venture of Abbott Laboratories is gratefully acknowledged. Michael Vrkljan wishes to thank Abbott Laboratories for a Summer Undergraduate Research Fellowship. The technical assistance of Gabe Evans and Emily Evans is gratefully acknowledged.

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