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Report

Physical Stability of a Recombinant Alpha$_1$-Antitrypsin Injection

Cheng-Der Yu,[1,2] Niek Roosdorp,[1] and Shamim Pushpala[1,3]

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Physical stability of a recombinant alpha$_1$-antitrypsin (rAAT) injection solution, namely, loss of rAAT to particulate formation was studied. The rAAT injection solution (1.0 mg/ml, pH 7.5, triple buffer of phosphate, Tris, and glycine, 0.075 *M* each) was filtered through a 0.2-μm filter and packaged in individual vials with rubber stoppers and aluminum seals. The vials were stored at 90, 80, 60, 45, 35, and 25°C, and samples taken at predetermined time intervals for each storage condition. Each sample was then filtered through a 0.2-μm filter to remove particulates. The filtrate was then assayed for total protein by the biuret–phenol method. A typical first-order loss of rAAT was observed. Data were fitted to a first-order kinetics, and the shelf life (defined as t_{90}, the time for the product to reach 90% remaining) was calculated from the resultant rate constant. The shelf lives were plotted against reciprocals of storage temperatures (a modified Arrhenius plot). A linear regression line ($r = 0.9831$) was drawn through the data points and extrapolated to 4°C. At 4°C, the predicted shelf life of the rAAT solution is 5.1 months. In real life, a batch of the rAAT solution showed that 61% of the rAAT remained in the filtrate after 18 months of storage at 4°C. The observed stability compares fairly well with the predicted value of 69% for the same duration of storage at 4°C. From the slope of the linear regression line, the activation energy for the particulate formation of the rAAT was calculated to be 19 kcal. This value is comparable to the activation energy of 20 kcal for ovalbumin denaturation reaction reported by Simpson and Kauzmann.

KEY WORDS: alpha$_1$-antitrypsin (rAAT); rAAT physical stability; particulate formation; protein aggregation; shelf-line prediction.

INTRODUCTION

Alpha$_1$-antitrypsin (AAT) is known to be very labile in solutions (1,2). For example, Lieberman (1) reported an *in vitro* half-life of 60 min for normal plasma-derived AAT at 55°C as measured by its trypsin-inhibitory capacity. In the same study, he reported that half-lives for other phenotype AATs were about 30 min. Recombinant AATs (358 methionine and 358 valine) were reported (2) to have an *in vitro* half-life of 40 min at 56°C and an *in vivo* (rabbits) half-life of 8.5 hr. The rapid loss of AAT activity was attributed to oxidation of the exposed methionine residue (2) and a general thermal lability (1) of the AAT. Oxidation and/or thermal inactivation of a protein are often accompanied by a conformational change of the protein molecule. The conformational change frequently results in aggregation, particulate formation, or precipitation. An AAT solution may therefore encounter physical stability problems in addition to the problem of activity loss. Efforts are warranted to ensure an adequate physical shelf-life in addition to the required potency shelf-life for an injectable rAAT formulation. This study represents an initial investigation of the physical stability of the rAAT injection solution upon storage. The scope of the study was limited to the assessment of the particulate formation problem for the alpha$_1$-antitrypsin injection. A separate study will investigate the kinetics of dimerization and polymerization of alpha$_1$-antitrypsin and the activities of various species of the aggregated alpha$_1$-antitrypsin.

MATERIALS AND METHODS

Alpha$_1$-Antitrypsin

The recombinant alpha$_1$-antitrypsin used in this study was the yeast mutant 358 methionine AAT. The protein was purified from yeast cell lysate using ion-exchange, affinity, and size-exclusion chromatography. The purified protein was then lyophilized and stored at 4°C until use. The lyophilized protein was 91.7% active and consisted of 95.2% monomers and 4.8% dimers.

Buffer Solution

A triple buffer (Tris, 0.075 *M;* glycine, 0.075 *M;* sodium phosphate, 0.075 *M;* pH 7.5) was freshly made and filtered through a 0.2-μm filter prior to use.

Physical Stability Study

A solution of rAAT (1.0 mg/ml) was made by dissolving the lyophilized rAAT powder in the triple buffer (pH 7.5). The solution was filtered through a 0.2-μm filter and subdi-

[1] Cooper Laboratories Inc., Mountain View, California 94043.
[2] To whom correspondence should be addressed.
[3] Present address: Institute of Pharmaceutical Sciences, Syntex Research, Palo Alto, California 94304.

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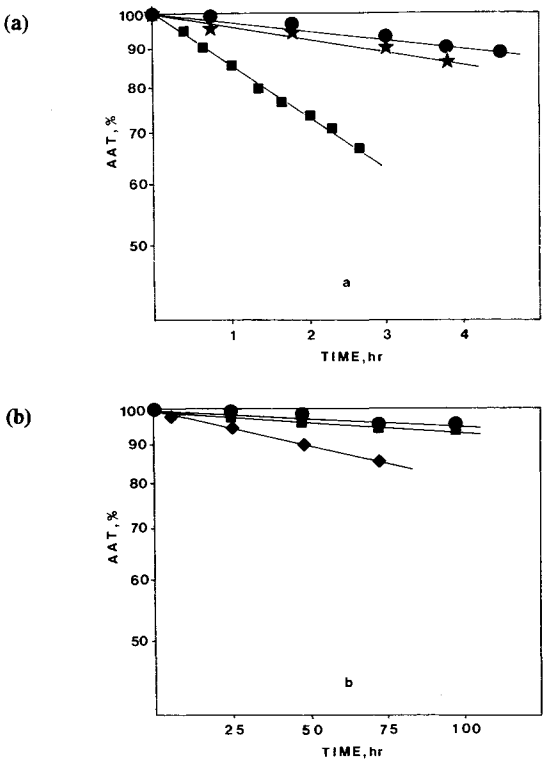

Fig. 1. First-order loss of rAAT to precipitation. (a) At 90°C (■), 80°C (★), and 60°C (●). (b) At 45°C (♦), 35°C (■), and 25°C (●).

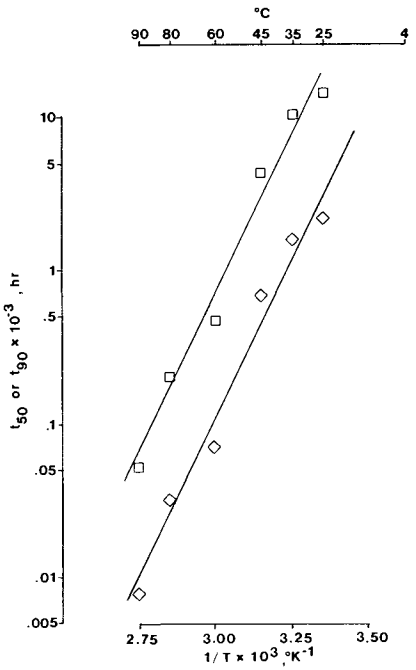

Fig. 2. Half-lives (□) and shelf lives (◇) of rAAT at various storage temperatures.

vided into 0.5-ml aliquots. The filter membrane used was a low-protein binding polyvinylidene difluoride membrane (Millex GV, Millipore, Bedford, Mass.). No protein binding problem was encountered. Each aliquot was filled into a small vial and capped with a rubber stopper and an aluminum seal. A given number of vials were put in an oven at 25, 35, 45, 60, 80, or 90°C. A vial was pulled out from each oven at a predetermined time interval. The content of the vial was filtered through a 0.2-μm filter to remove the particulates. The filtrate was then assayed for total soluble protein by the biuret–phenol method (3).

RESULTS AND DISCUSSION

Figures 1a and b show semi-log plots of the percentage rAAT remaining versus time for a given storage temperature. A typical first-order loss of rAAT was observed. Data were fitted to a first-order kinetics and a linear regression line was drawn through the data points. The half-life (t_{50}; defined as the time for the product to reach 50% remaining) and the shelf life (t_{90}; defined as the time for the product to reach 90% remaining) were calculated from the resultant rate constants, as shown in Table I. Also shown in Table I are the coefficients of correlation (r) for the linear regression. The half-lives and the shelf lives were then plotted against the reciprocals of storage temperatures (a modified Arrhenius plot) as shown in Fig. 2. For the ease of data interpretation, temperatures as degrees centigrade corresponding to reciprocals of temperatures expressed as degrees kelvin are also indicated in Fig. 2. A linear regression line (r = 0.9831) was drawn through the data points and extrapolated to 4°C. At 4°C, the predicted shelf life of the rAAT injection is 5.1 months. In real life, a batch of the rAAT solution showed that 61% of the rAAT remained in the filtrate after 18 months of storage at 4°C. The observed stability compares fairly well with the predicted value of 69% for the same duration of storage at 4°C. The physical half-lives of the rAAT injection at various storage temperatures are also shown in Fig. 2. At 56°C, the half-life was calculated to be 102 hr, which was quite different from the reported (2) activity half-life of 40 min at the same temperature. The rate of loss of rAAT activity is obviously much faster than the rate of loss of rAAT to particulate formation. Nevertheless, Travis *et al.* (2) reported that the activity of rAAT can be preserved with some success in a solution of 0.2 *M* mercaptoethanol (a reducing

Table I. Calculated Half-Lives and Shelf Lives at Various Storage Temperatures

	Storage temperature (°C)					
	90	80	60	45	35	25
Correlation coefficient (r)	0.986	0.970	0.960	0.944	0.969	0.983
Rate constant × 10^3 (hr^{-1})	138	34.3	15.0	1.59	0.68	0.50
Half-life (t_{50}, hr)	5.02	20.2	46.2	436	1017	1383
Shelf-life (t_{90}, hr)	0.76	3.07	7.02	66.3	154.7	210.3

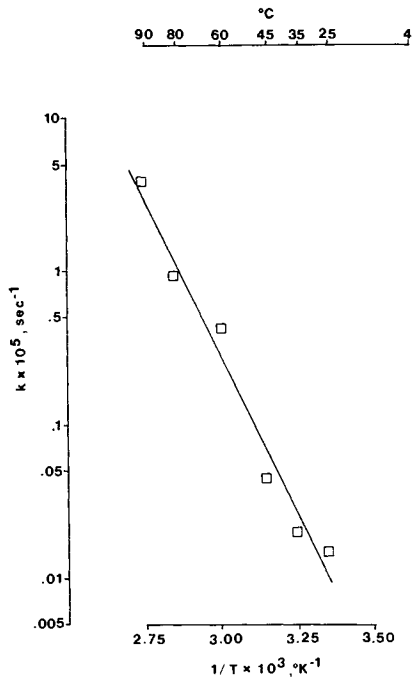

Fig. 3. Arrhenius plot of thermal denaturation of rAAT in solution.

agent). They also reported (2) that the inactivated rAAT could be reverted to 80% active rAAT by dialysis against 0.2 *M* mercaptoethanol. It is likely that the 20% unrecoverable AAT activity is lost to the irreversible physical changes of AAT such as aggregation, particulate formation, and precipitation.

An Arrhenius plot of rate constants versus reciprocals of storage temperatures is shown in Fig. 3. From the slope of the linear regression line, the activation energy for the physical changes of the rAAT injection was calculated to be 19 kcal/mol. This value is quite comparable to the activation energy of 20 kcal/mol for ovalbumin denaturation reaction reported by Simpson and Kauzmann (4). It is possible that the physical changes observed in this study are due predominantly to protein denaturation.

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