

## **SUPPLEMENTARY MATERIAL**

### ***Deciphering the role of trehalose in hindering antithrombin polymerization***

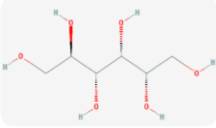
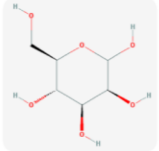
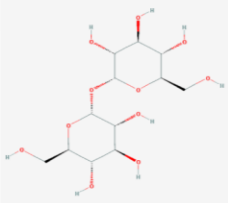
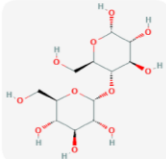
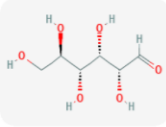
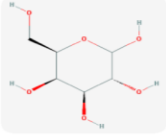
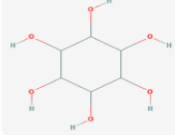
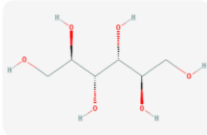
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Mohamad Aman Jairajpuri<sup>a,\*</sup>

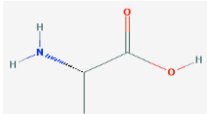
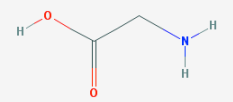
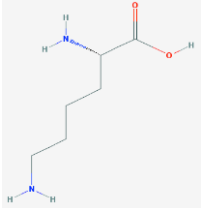
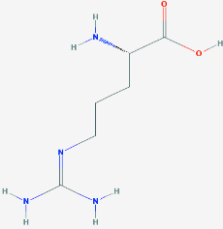
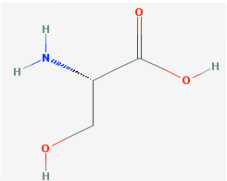
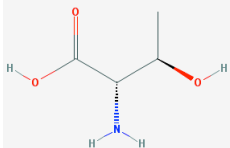
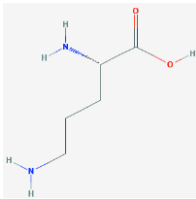
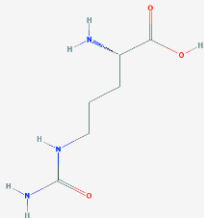
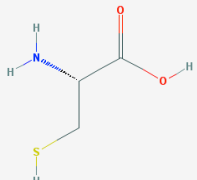
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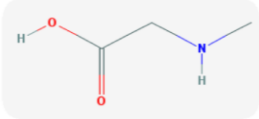
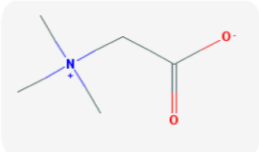
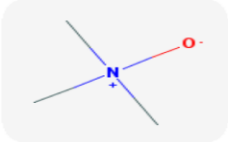
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**Table S1:** List of small molecules used in *in vitro* screening.

	<b>Sugars and Polyols</b>	<b>CID No.</b>	<b>Chemical structure</b>
<b>1</b>	Sorbitol	5780	 The chemical structure of sorbitol is shown as a linear chain of six carbon atoms. Each carbon is bonded to hydroxyl groups (-OH) and hydrogen atoms (-H). The hydroxyl groups are positioned at the 1, 2, 3, 4, and 6 positions, while hydrogen atoms are at the 2, 3, 4, and 5 positions. The structure is drawn in a perspective view with red oxygen atoms and white hydrogen atoms.
<b>2</b>	Mannose	18950	 The chemical structure of mannose is shown as a six-membered pyranose ring. It has hydroxyl groups (-OH) at the 2, 3, and 6 positions, and hydrogen atoms (-H) at the 4 and 5 positions. The structure is drawn in a perspective view with red oxygen atoms and white hydrogen atoms.
<b>3</b>	Trehalose	7427	 The chemical structure of trehalose is shown as two six-membered pyranose rings linked together by an alpha-1,4-glycosidic bond. Each ring has hydroxyl groups (-OH) and hydrogen atoms (-H) at various positions. The structure is drawn in a perspective view with red oxygen atoms and white hydrogen atoms.
<b>4</b>	Maltose	439341	 The chemical structure of maltose is shown as two six-membered pyranose rings linked together by an alpha-1,4-glycosidic bond. Each ring has hydroxyl groups (-OH) and hydrogen atoms (-H) at various positions. The structure is drawn in a perspective view with red oxygen atoms and white hydrogen atoms.
<b>5</b>	Dextrose	22814120	 The chemical structure of dextrose is shown as a linear chain of six carbon atoms. It has hydroxyl groups (-OH) at the 2, 3, and 6 positions, and hydrogen atoms (-H) at the 4 and 5 positions. The structure is drawn in a perspective view with red oxygen atoms and white hydrogen atoms.
<b>6</b>	Galactose	6036	 The chemical structure of galactose is shown as a six-membered pyranose ring. It has hydroxyl groups (-OH) at the 2, 3, and 6 positions, and hydrogen atoms (-H) at the 4 and 5 positions. The structure is drawn in a perspective view with red oxygen atoms and white hydrogen atoms.
<b>7</b>	Inositol	892	 The chemical structure of inositol is shown as a six-membered pyranose ring. It has hydroxyl groups (-OH) at the 1, 2, 3, 4, and 6 positions, and a hydrogen atom (-H) at the 5 position. The structure is drawn in a perspective view with red oxygen atoms and white hydrogen atoms.
<b>8</b>	Mannitol	6251	 The chemical structure of mannitol is shown as a linear chain of six carbon atoms. It has hydroxyl groups (-OH) at the 1, 2, 3, 4, and 6 positions, and hydrogen atoms (-H) at the 2, 3, 4, and 5 positions. The structure is drawn in a perspective view with red oxygen atoms and white hydrogen atoms.

	Amino Acids	CID No.	Chemical structure
1	Alanine	5950	 The chemical structure of Alanine shows a central alpha-carbon bonded to a hydrogen atom, an amino group (NH2), a methyl group, and a carboxyl group (COOH).
2	Glycine	750	 The chemical structure of Glycine shows a central alpha-carbon bonded to two hydrogen atoms, an amino group (NH2), and a carboxyl group (COOH).
3	Lysine	5962	 The chemical structure of Lysine shows a central alpha-carbon bonded to a hydrogen atom, an amino group (NH2), a carboxyl group (COOH), and a long aliphatic side chain ending in a primary amino group (NH2).
4	Arginine	6322	 The chemical structure of Arginine shows a central alpha-carbon bonded to a hydrogen atom, an amino group (NH2), a carboxyl group (COOH), and a long aliphatic side chain ending in a guanidino group.
5	Serine	5951	 The chemical structure of Serine shows a central alpha-carbon bonded to a hydrogen atom, an amino group (NH2), a carboxyl group (COOH), and a hydroxymethyl side chain (-CH2OH).
6	Threonine	6288	 The chemical structure of Threonine shows a central alpha-carbon bonded to a hydrogen atom, an amino group (NH2), a carboxyl group (COOH), and a side chain with a methyl group and a hydroxyl group.
7	Ornithine	6262	 The chemical structure of Ornithine shows a central alpha-carbon bonded to a hydrogen atom, an amino group (NH2), a carboxyl group (COOH), and a long aliphatic side chain ending in a primary amino group (NH2).
8	Citrulline	9750	 The chemical structure of Citrulline shows a central alpha-carbon bonded to a hydrogen atom, an amino group (NH2), a carboxyl group (COOH), and a long aliphatic side chain ending in a primary amino group (NH2).
9	Cysteine	5862	 The chemical structure of Cysteine shows a central alpha-carbon bonded to a hydrogen atom, an amino group (NH2), a carboxyl group (COOH), and a side chain with a thiol group (-SH).

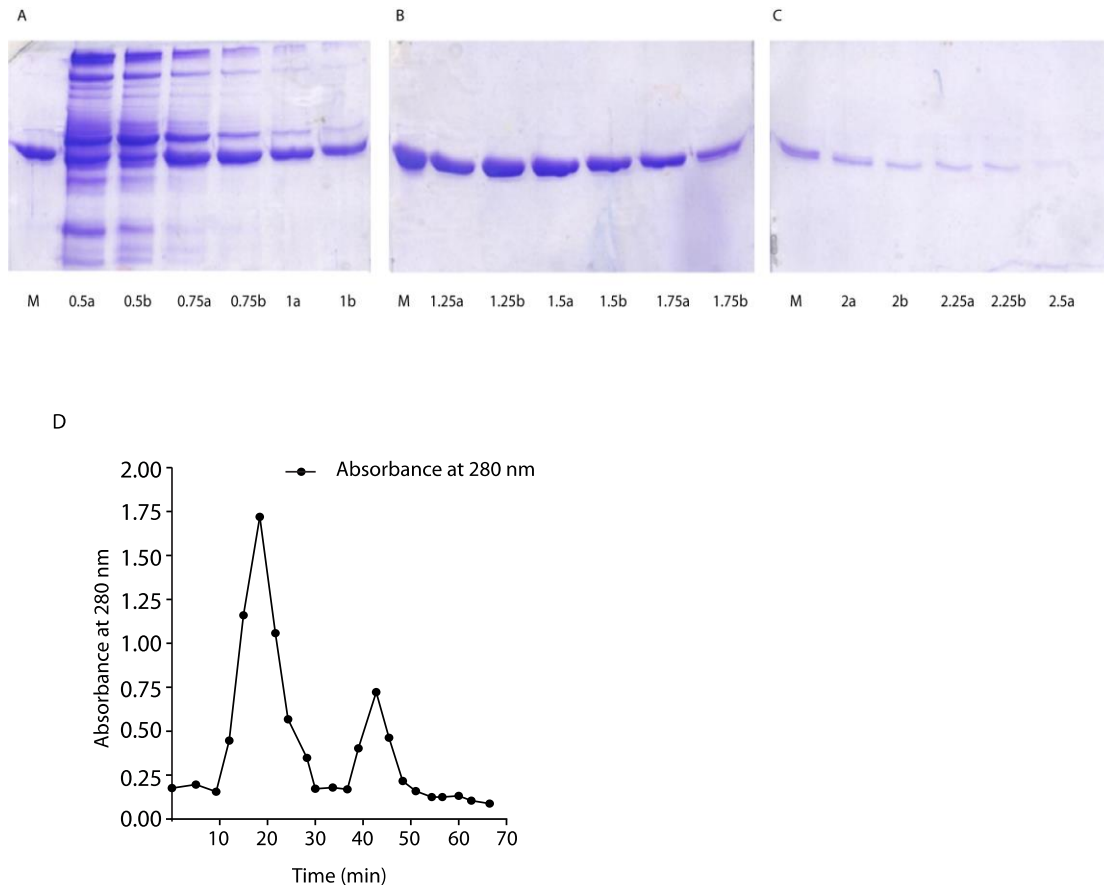
	<b>Methylamines</b>	<b>CID No.</b>	<b>Chemical structure</b>
<b>1</b>	Sarcosine	1088	 <p>The chemical structure of Sarcosine is shown as a skeletal structure. It consists of a central carbon atom double-bonded to an oxygen atom (red) and single-bonded to a hydroxyl group (red oxygen, white hydrogen) and a methylamino group (blue nitrogen with one white hydrogen, and a methyl group represented by a black line).</p>
<b>2</b>	Betaine	247	 <p>The chemical structure of Betaine is shown as a skeletal structure. It features a central carbon atom double-bonded to an oxygen atom (red) and single-bonded to a methyl group (black line) and a trimethylammonium group (blue nitrogen with a positive charge and three black lines representing methyl groups).</p>
<b>3</b>	Trimethylamine N-oxide (TMAO)	1145	 <p>The chemical structure of Trimethylamine N-oxide (TMAO) is shown as a skeletal structure. It consists of a central blue nitrogen atom with a positive charge, bonded to three black lines representing methyl groups and one red oxygen atom with a negative charge.</p>

**Table S2:** Secondary structure content of AT denatured with 2M GdnHCl in the absence and presence of 1M trehalose. Helical content was calculated as described [1].

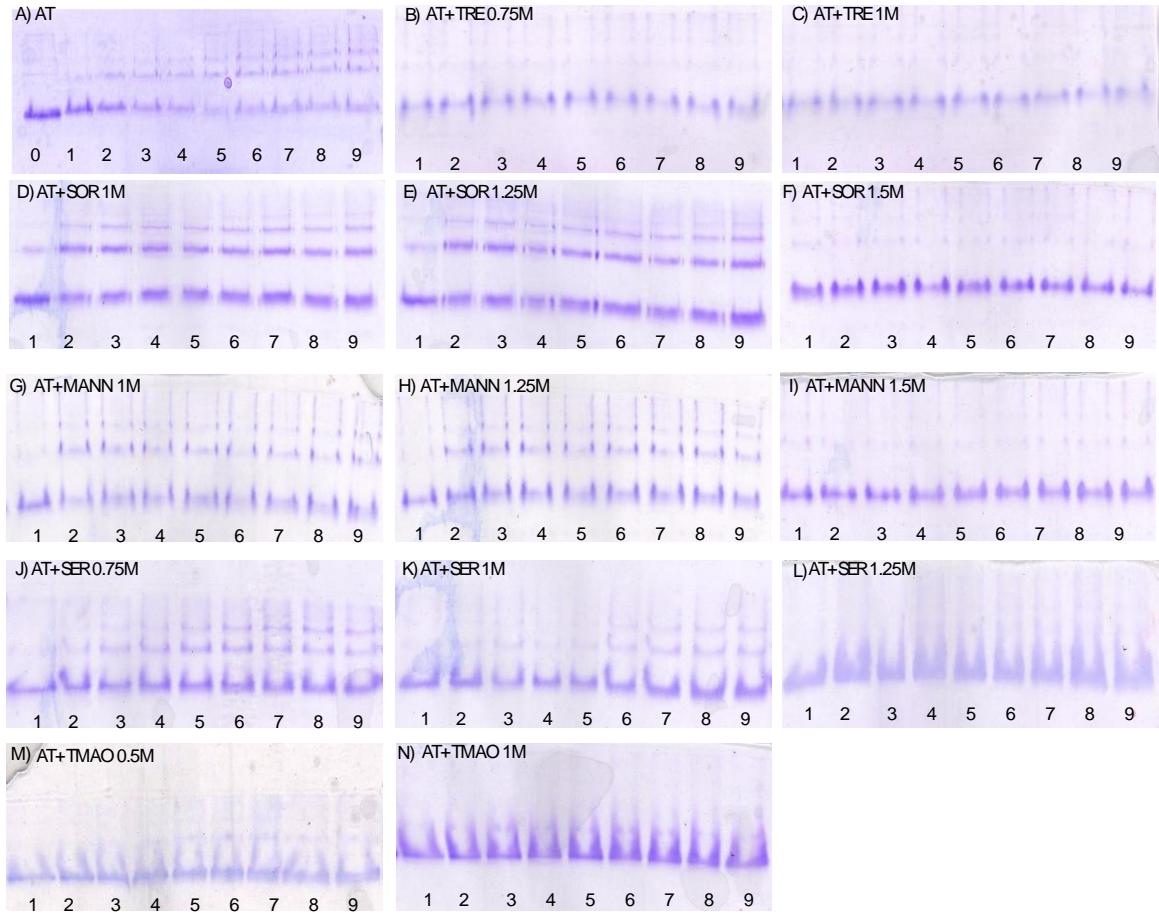
<b>Molecule</b>	<b>MRE<sub>222nm</sub></b>	<b>%alpha helix</b>
AT	- 4.3852	15.6 ± 0.03
AT+TRE	- 5.2993	21.7 ± 0.05

**Table S3:** Alteration of secondary structure of AT in the presence of trehalose. Secondary structures were calculated using the online software k2D [1,2]

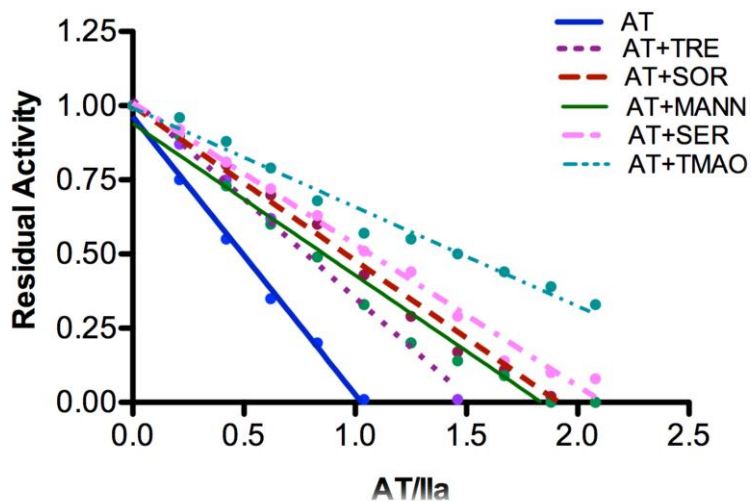
<b>Secondary Structure</b>	<b>AT</b>	<b>AT+TRE</b>
α-helix (%)	29	36
β-sheets (%)	22	20
Random Coil (%)	49	43



**Figure S1: Purification of AT from human plasma.** Elution profile of purified AT is shown. (A-C) SDS-PAGE of AT purified using a salt gradient of NaCl (20 mM phosphate buffer containing 100mM NaCl, 0.1mM EDTA, pH 7.4 and ionic strength 0.15). Elution of protein started from 0.15M NaCl upto 2.5M NaCl. (A) Lanes showing the fractions collected from 0.5M upto 1M NaCl gradient run in doublets as indicated. (B) Fractions collected from 1.25M upto 1.75M are shown as in A. (C) Fractions from 2M upto 2.5M NaCl are shown as in panels A and B. Fractions containing 0.15-0.5 M gradients were treated as wash. Fractions showing single bands of purified protein were pooled, desalted and used in the study. M denotes pure AT run as marker. Lanes denotes different concentrations of NaCl as indicated. (D) Absorbance profile of the eluted fractions is shown. Each fraction was eluted for 3 minutes and absorbance was read at 280nm.

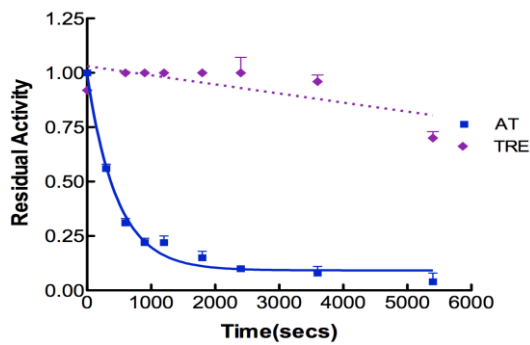


**Figure S2: Concentration dependence of small molecules that successfully retarded AT polymerization.** Polymers of AT were prepared by heating  $100\mu\text{g ml}^{-1}$  of native AT in total of 1ml at  $60^{\circ}\text{C}$  in 50 mM Tris and 50 mM KCL buffer, pH 7.4 in the absence (A) and presence of small molecules (B-N) at different time intervals. Samples were removed at indicated times, snap frozen and stored at  $-80^{\circ}\text{C}$  for analysis on Native-PAGE. Concentration dependence of trehalose (B-C); sorbitol (D-F); Mannose (G-I); Serine (J-L) and TMAO (M-N) is shown. Lane 0 indicates pure AT protein, lane 1-9 indicates time 0, 5, 10, 15, 20, 45, 60, 75 and 90 minutes of incubation respectively.

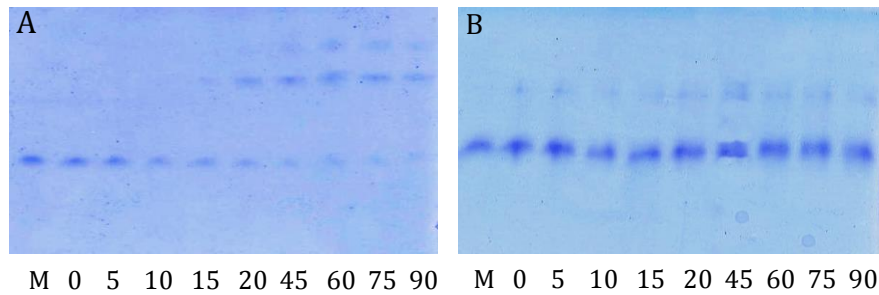


**Figure S3: Stoichiometries of thrombin (IIa) inhibition by AT in the absence and presence of small molecules.** Stoichiometries of thrombin inhibition were determined from residual protease activities with increasing AT concentrations as described previously (2). Briefly, 0–1000nM AT and 400nM of thrombin (IIa) in PNE-PEG buffer (20mM phosphate containing 100mM NaCl, 0.1mM EDTA and 0.1% polyethylene glycol 6000) were incubated at 25 °C in the absence and presence of 1M trehalose, 1.5M sorbitol, 1.5M mannose, 1.25M serine and 1.25M TMAO. Residual enzyme activities were measured by adding 0.15 mM thrombin substrate S-2238 and recording the initial hydrolysis rate at 405 nm. SI's were taken as the x-intercept of the linear regression when residual protease activity is plotted against the ratio of AT to thrombin. Appropriate thrombin and S2238 controls/blanks with small molecules in the absence of protein were taken.





**Figure S4: Kinetics of polymer transition in the absence and presence of trehalose were assessed under polymerization conditions.** Briefly, 100  $\mu\text{g/ml}$  of native AT in a total of 1 ml was incubated at  $60^\circ\text{C}$  in PNE buffer, pH 7.4, in the absence and presence of trehalose. Samples were removed at indicated times and were assayed for thrombin progressive activity (in PNE-PEG buffer) to assess the loss of AT inhibitory activity due to transition to polymeric AT with time. Reaction for the measurements of activity was set up under pseudo first order condition and contained AT and thrombin in a 10:1 ratio. AT and thrombin were reacted in microplates, and following the Enzyme + Inhibitor incubations, S-2238 substrate was added and measured at 405 nm. Appropriate thrombin and S-2238 controls with trehalose in the absence of AT were taken.



**Figure S5:** Native PAGE Gel representing the heat induced polymerization of AT (2 $\mu$ M) in the presence of (A) 10mM Trehalose Octasulfate and (B) 50mM Trehalose Octasulfate

#### Supplementary references:

- [1] Crevenna AH, Naredi-Rainer N, Lamb DC, Wedlich-Söldner R, Dzubiella J. (2012) Effects of Hofmeister ions on the  $\alpha$ -helical structure of proteins. *Biophysical Journal* **102(4)**:907-15.
- [2] Andrade, M. A., Chacón, P., Merelo, J.J., Moran, F. (1993). "Evaluation of secondary structure of proteins from UV circular dichroism spectra using an unsupervised learning neural network." *Protein Eng* **6(4)**: 383-890.
- [3] Jairajpuri, M. A., Lu, A., Desai, U., Olson, S. T., Bjork, I., and Bock, S. C. (2003) Antithrombin III phenylalanines 122 and 121 contribute to its high affinity for heparin and its conformational activation. *The Journal of biological chemistry* **278**, 15941-15950