Prometheus Protocol PR-P-015

Thermal Stability and Aggregation of Insulin

Insulin is a peptide hormone produced by the pancreatic islets consisting of an A-chain (21 residues) and a B-chain (30 residues) which are linked by two disulfide bonds. It is present at very low concentrations in the bloodstream and regulates the metabolism of carbohydrates, fats and protein. In the presence of zinc atoms, human insulin self-associates to hexamers which is physiologically useful, as the relatively small size of the hexamer allows for dense packing within the secretory granulae of the pancreatic beta-cells. However, since the hexamers are too large for diffusion across the cell membrane, dissociation of the hexamers is a prerequisite for absorption into the circulation. Insulin has no tryptophans, but four tyrosines.

thermal unfolding | tyrosine fluorescence | T_m | T_{agg}

A1. Target/Fluorescent Molecule

Insulin
uniprot.org/uniprot/P01308

A2. Molecule Class/Organism

Peptide hormone
Homo sapiens (Human)

A3. Sequence/Formula

A-chain
GIVEQCCTSI CSLYQLENYC N

B-chain
FVNQHLCGSH LVEALYLVCG ERGFFYTPKT

A4. Purification Strategy/Source

Recombinant, expressed in yeast
Sigma-Aldrich
I2643

A5. Stock Concentration/Stock Buffer

5 mg/mL | 0.86 mM
Phosphate buffered saline (PBS), pH 7.4

A6. Molecular Weight/Extinction Coefficient

5807 Da
6,200 $M^{-1}cm^{-1}$ (ε_{276})

A7. Dilution Buffer

7 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4, 100 mM NaCl, 2 mM KCl, 1.2 mM EDTA (or 0.6 mM $ZnCl_2$)

D1. nanoDSF System/Capillaries

Prometheus NT.48 (NanoTemper Technologies GmbH)
Prometheus Aggregation Detection Optics (PR-AGO, NanoTemper Technologies GmbH)
Prometheus High Temperature Upgrade (PR-HTU, NanoTemper Technologies GmbH)
High Sensitivity Capillaries Prometheus NT.48 nanoDSF Grade (PR-C006, NanoTemper Technologies GmbH)
Capillary Sealing Paste Prometheus Series (PR-P001, NanoTemper Technologies GmbH)
Capillary Sealing Applicators Prometheus Series (PR-P002, NanoTemper Technologies GmbH)

D2. nanoDSF Software

PR.ThermControl v2.1 | PR.TimeControl v1.0.2 | PR.StabilityAnalysis v1.1 (NanoTemper Technologies GmbH)
nanotempertech.com/prometheus-software

D3. nanoDSF Experiment

PR.ThermControl

1. Prepare 20 µL of a 3.2 mM $ZnCl_2$ solution and 20 µL of a 6.4 mM EDTA solution in ddH_2O.
2. Add 80 µL of 0.86 mM insulin in PBS to each of the tubes to obtain 100 µL of 0.6 mM insulin solutions containing 0.6 mM **$ZnCl_2$** and 1.2 mM **EDTA**, respectively.

Tube	Volume	Insulin	Buffer	Additive
1	100 µL	0.6 mM	7 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4, 100 mM NaCl, 2 mM KCl	1.2 mM EDTA
2	100 µL	0.6 mM	7 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4, 100 mM NaCl, 2 mM KCl	0.6 mM $ZnCl_2$

3. Completely load three capillaries from each of the two tubes, place them on positions 1 – 6 of the capillary tray, and place the magnetic lid to fix the capillaries.
4. Start a new session of the *PR.Therm Control* software, go to 'Melting Scan' and prepare a run with the following settings:
    a. Capillaries 1 – 6 selected
    b. 1.0°C/min
    c. 25°C – 110°C
    d. 10% excitation power
5. Seal the capillaries with capillary sealing paste and repeat sealing after 5 minutes.
6. Start the measurement.

PR.TimeControl

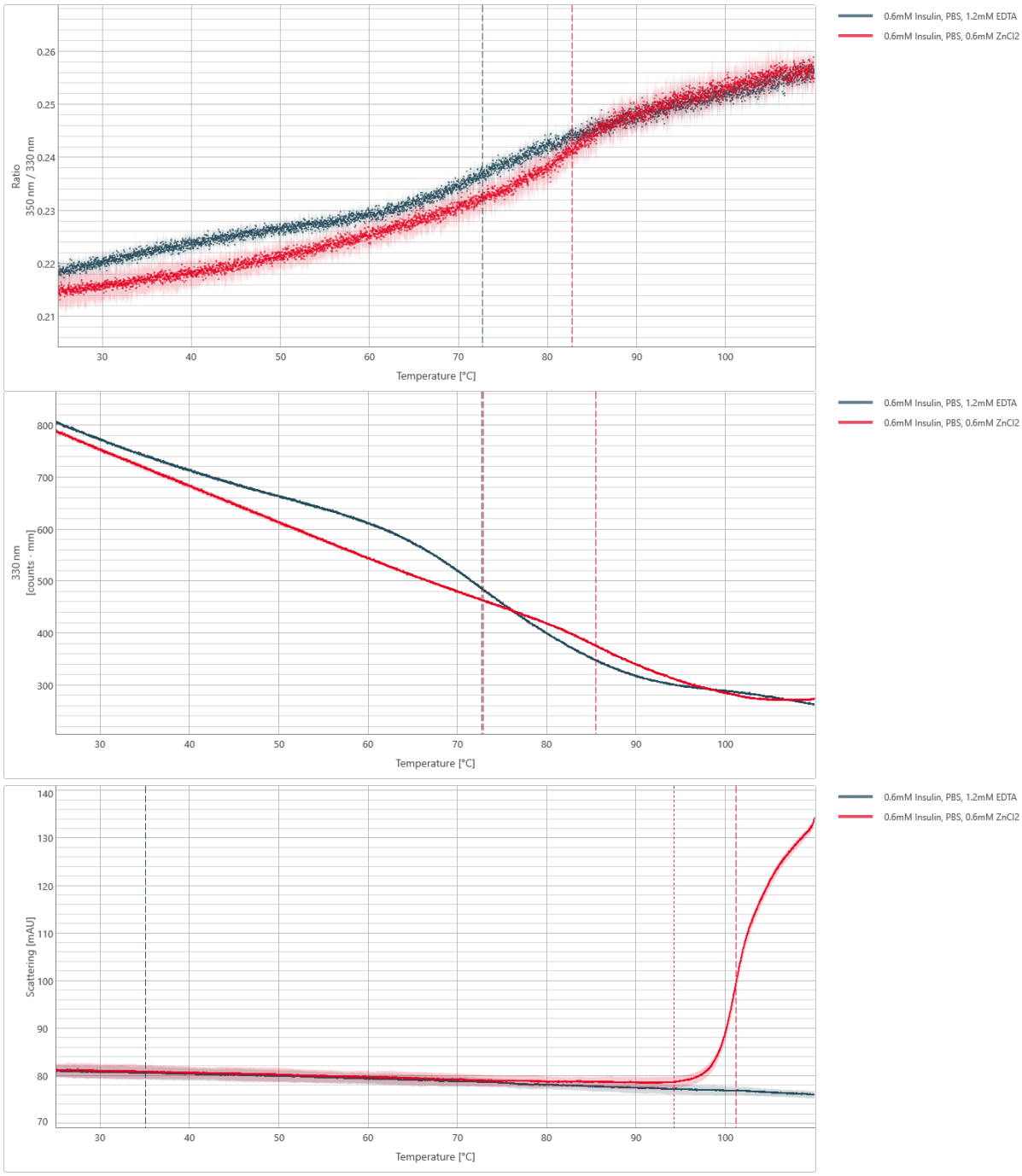
1. Start a session of the *PR.TimeControl* software.
2. Change the Thermostat Set Point to 60°C by using the touch display on the instrument and wait for the system to reach this temperature.
3. Go to 'Measurement Scan' and prepare a run with the following settings:
    a. Capillaries 1 – 6 selected
    b. Isothermal
    c. 72°C
    d. 180 min
    e. 10% excitation power
4. Completely load three capillaries from each of the two tubes of step 2, place them on positions 1 – 6 of the capillary tray, and place the magnetic lid to fix the capillary.
5. Seal the capillaries with capillary sealing paste and repeat sealing after 5 minutes.

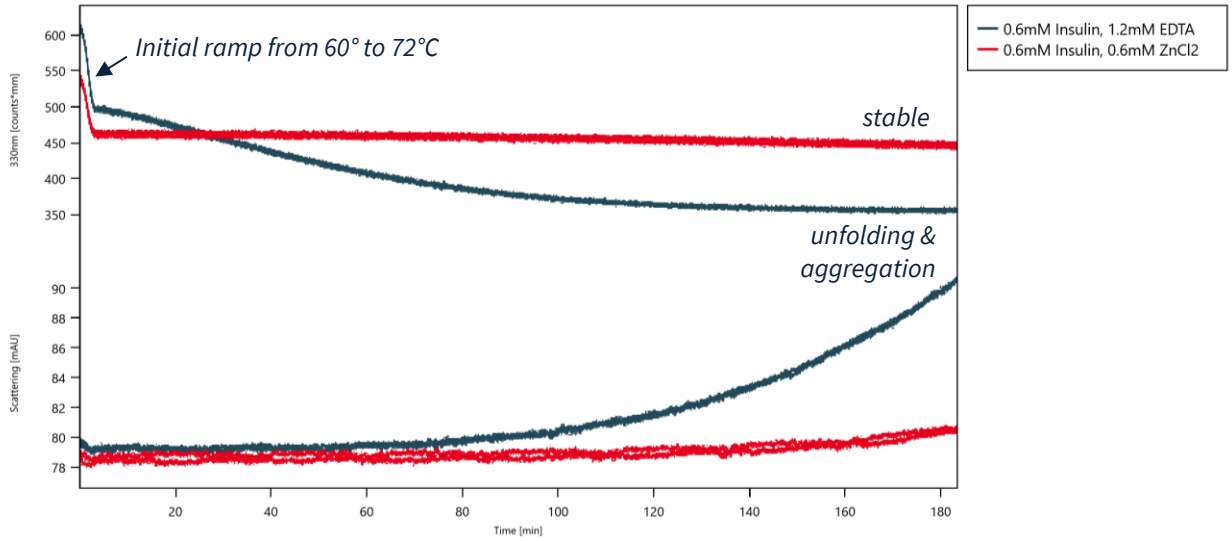
6. Start the measurement.

D4. nanoDSF Results

– Zn: T_m = 72.7°C ± 0.4°C

+ Zn: T_m = 82.8°C ± 0.6°C | T_{agg} = 94.2°C ± 0.9°C *Stabilization, but also strong aggregation*





Slow denaturation of Zn-free insulin while Zn-insulin remains stable
Aggregation of Zn-free insulin after 2 hours at 72°C

D5. Reference Results/Supporting Results

– Zn^{2+}: T_m = ~55°–70°C Tyrosine Autofluorescence and Circular Dichroism (CD)
Bekard et al., Biophys J, 2009, 97 (9), 2521-2531

– Zn^{2+}: T_m = ~68.7°C Differential Scanning Calorimetry (DSC)
+ Zn^{2+}: T_m = ~88.0°C

Zinc-free insulin, which is primarily dimeric at room temperature, unfolded at ~70 °C. […] Small amounts of zinc caused a biphasic thermal denaturation pattern of insulin. The biphasic denaturation is caused by a redistribution of zinc ions during the heating process and results in two distinct transitions with T_m's of ~70 and ~87 °C corresponding to monomer/dimer and hexamer, respectively. At high zinc concentrations (5 Zn^{2+} ions/hexamer), only the hexamer transition is observed.
Huus et al., Biochemistry, 2005, 44 (33), 11171–11177

The insulin monomer is less stable than the hexamer and tends to aggregate.
Much greater aggregation rate for the Zn-insulin hexamer relative to the Zn-free dimer.
Xu et al., Langmuir 2012, 28, 579–586

E. Contributors

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