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Article

Cold-Active Starch-Degrading Enzymes from a Cold and Alkaline Greenland Environment: Role of Ca^{2+} Ions and Conformational Dynamics in Psychrophilicity

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Abstract: Cold-active enzymes hold promise in energy-efficient processes. Amylases are widely used in household and industrial applications, but only a few are cold-active. Here we describe three novel secreted amylases Rho13, Ika2 and I3C6, all from bacteria growing in the cold and alkaline ikaite columns in Greenland. They all hydrolysed starch to smaller malto-oligomers, but only Rho13 and Ika2 hydrolysed cyclodextrins and only Ika2 displayed transglycosylation activity. Ika2 forms a stable dimer, while both Rho13 and I3C6 are mainly monomeric. They all have optimal temperatures around 30–35°C and significant activity below 20°C, but Rho13 and I3C6 had an alkaline pH optimum while Ika2 was markedly acidophilic. They show complex dependence on Ca^{2+} concentration, with activity of Rho13 and I3C6 following a bell-shaped dependence and Ika2 being unaffected; however, removal of Ca^{2+} reduces stability of all 3 enzymes. Loss of structure occurs well above the optimal activity, showing the characteristic psychrophilic divorce between activity and stability. MD simulations showed that Ika2 did not have a well-defined Ca^{2+} binding site, while Rho13 and I3C6 both maintain one stably bound Ca^{2+} ion. We identified psychrophilic features as higher levels of backbone fluctuations compared to mesophilic counterparts, founded in a lower number of internal hydrogen bonds and salt bridges. This increased fluctuation is also found in regions outside the active site and may provide easier substrate access and accommodation and faster barrier transitions. Our work sheds further light on the many ways in which psychrophilic enzymes adapt to increased catalysis at lower temperatures.

Keywords: psychrophilic enzymes; protein stability; molecular dynamics

1. Introduction

Exploring cold environments for new cold-active enzymes can lead to the discovery of novel enzyme variants with unique properties and functionalities. These enzymes may exhibit distinct

substrate specificities, thermal stabilities, or catalytic efficiencies, offering opportunities for tailored enzyme engineering and customization to specific industrial applications. The ability of cold-active enzymes to function effectively at low temperatures provides flexibility in process design. Industrial processes can be carried out at ambient temperatures or under refrigeration, eliminating the need for energy-intensive heating systems. This minimizes the risk of thermal degradation of sensitive substrates or products and reduces operational costs. Novel energy-efficient processes are required in industry as well as in private households to contribute to a more sustainable society. One contribution to this reduction of emission is to apply truly cold-active enzymes to detergents to make washing at low temperatures even more efficient.

Cold-active enzymes for detergents are already industrially applied today. However, most of these enzymes display optimal activity at temperatures between 20 and 45°C [1] with marginal activity at 5-20°C. This is usually compensated for by over-dosing the amount of enzyme used. To move beyond these simplistic solutions, we need to discover novel, truly cold-active enzymes from natural environments that provide in-depth knowledge on cold-activity [2]. This knowledge can then be mobilized to either exploit these new enzymes directly or modify currently used industrial enzymes to increase their activity at low temperatures and to reduce the amount of enzyme required.

α -amylases (EC 3.2.1.1) are a major class of enzymes used in laundry detergents, as well as in production of textiles, biofuel, feed, food, and beverages. They catalyze the hydrolysis of α -1,4 glycosidic bonds in starch and starch-related polysaccharides including amylopectin, amylose, cyclodextrins, glycogen, and pullulan [3–5]. Amylases are furthermore relevant for bioremediation. Cold-active amylases are particularly interesting because of their ability to degrade organic matter in e.g. waste treatment in cold and temperate regions [6]. Some cold-active amylases even possess higher specific activity than their meso- or thermophilic counterparts [6].

α -amylases are structurally grouped in four different glycoside hydrolase (GH) families: GH13, GH57, GH119, and GH126, (CAZy.org). The largest is GH13, whose members form a $(\beta/\alpha)_8$ barrel structure and a conserved catalytic triad consisting of two Asp and one Glu, or in a few cases one Asp, one Glu and 1 His [7]. Besides α -amylases, GH13 contains endo- as well as exo-acting amylase-family enzymes with a wide range of different substrate specificities, including pullulanases (catalysing hydrolysis of (1→6)- α -D-glucosidic linkages in pullulan, amylopectin and glycogen), neopullulanases (α -amylases specifically catalyzing the hydrolysis of α -1,4-glucosidic bonds in pullulan), and cyclomaltodextrinases (catalyzing the linearization of cyclomaltodextrin into maltodextrin) [8–13].

An attractive feature of cold-active amylases is that they are active at moderate as well as low temperatures. Most known amylases are either meso- or thermophilic, and only a limited number of cold-active amylases is known. These include an α -amylase from the soil bacterium *Bacillus subtilis* [14], an α -amylase from the Himalayan soil bacterium *Bacillus cereus* [15], a salt-tolerant α -amylase from the marine bacterium *Zunongwangia profunda* [16], and the well-characterized α -amylase from the Antarctic marine bacterium *Alteromonas haloplanktis* A23, which shows optimal amylolytic activity at 30°C, and maintains 18% activity at 3°C [17–20].

Since most amylases are exported, they will be exposed to conditions which may differ from the cytosolic environment in terms of ionic strength or pH. Amylases with significant residual activity at low temperature have so far been found in aquatic environments, and all show relatively broad pH profiles, with optima around pH 10 (*Bacillus subtilis* N8 and *Bacillus cereus* GA6) and pH 7 (*Zunongwangia profunda* and *Alteromonas haloplanktis* A23). Alkaliphilic profiles are particularly useful for detergent enzymes, since detergent-based washing usually takes place around pH 9-10 [21].

To discover novel cold-active, alkali-stable amylases, we have investigated the only environment on Earth known to be both cold and alkaline, namely the ikaite columns in the Ikka Fjord, SW Greenland. These tufa columns, consisting of the meta-stable mineral ikaite (a hydrated form of calcium carbonate), are formed due to precipitation of calcium carbonate from highly alkaline spring water (pH 10.4) mixing with the cold seawater in the Ikka Fjord [22]. They are inhabited by diverse microbial communities [23]. Most of the isolated bacterial strains and recombinantly

expressed enzyme genes obtained from environmental DNA from the columns show significant extracellular enzymatic activity at high pH [24–26].

Here we identify and provide a detailed characterization of two novel, starch-degrading enzymes from these ikaite columns, namely Ika2 and Rho13. In parallel, we extend the characterization of I3C6, another amylase from the ikaite columns[27] to allow direct comparison of their enzymatic and biophysical properties with particular focus on their psychophilicity. I3C6 was identified in a metagenomic analysis rather than from functional screening of a single bacterial isolate[27] and had a reported pH optimum around 7-9 and a temperature optimum around 10-15°C, losing activity within 20 minutes at 45°C while retaining >70% of activity as low as 1°C, and with a pH optimum at pH 8-9. I3C6 activity was stimulated by Ca²⁺ but inhibited by Zn²⁺ and Cu²⁺. We have analysed product formation by pulsed amperometric detection to show endo-active release of maltose (G2) and larger oligosaccharides from amylopectin, amylose and hydrolyzed starch as well as hydrolysis of smaller maltooligosaccharides. The three enzymes also vary in their quaternary organization, pH-profiles and dependence on Ca²⁺ ions, but they all show loss of activity at temperatures well below the loss of structure. This characteristic psychophilic trait may be linked to their higher levels of backbone fluctuations when compared with mesophilic homologues. In turn, we propose that this provides easier substrate access and binding, as well as facilitating efficient conformational transitions across local minima during catalysis.

2. Materials and Methods

Identification of samples

All samples originate from the ikaite columns in the Ikka Fjord but were sampled at different times between 2006 and 2019, and the enzymes were detected and isolated by different approaches as described below.

Amylase Ika2: Samples of ikaite columns were collected by divers in the Ikka Fjord in the summer of 2019, under permission from the Government of Greenland (License no. G19-032), and stored at -20°C. To isolate bacteria from the ikaite columns, samples of 0.2-0.5 g of ikaite were drilled by hand from the interior of the column samples. The drill material was suspended in 1/10 strength R2 broth [per liter: 0.05 g yeast extract, 0.05 g Bacto peptone, 0.05 g Bacto casamino acids, 0.03 g sodium pyruvate, 0.03 g KH₂PO₄, 0.005 g MgSO₄ * 7 H₂O, 1 g NaCl] adjusted to pH 10 with 50 mM Na₂CO₃/NaHCO₃, and aliquots were used for culturing experiments. Bacterial strains from sampled ikaite material displaying amylase activity were isolated from agar plates with 1/10 strength R2 agar [R2 broth supplemented with 15 g/L agar, no glucose] adjusted to pH 10 and supplemented with 0.5 g/L AZCL-amylose (Megazyme). Plates were incubated at 5°C for 3 months, and colonies producing a halo of solubilized chromogenic amylose were re-streaked on the same substrate as above until strain purity was obtained.

Amylase RhoGH13_36 (denoted below): The gene sequence was identified by screening the genome sequence of the ikaite bacterium *Rhodanellum psychrophilum*[28,29] (NCBI accession number AWXR000000000) for putative amylase-encoding genes (see details below).

Amylase AmyI3C6 (denoted below): This enzyme was previously identified and described by Vester et al.[27] (acc. no. KJ790257).

Identification of amylase-coding genes and homology alignment

To identify the amylase-coding genes in the cultured isolate (Ika2), the strain was subjected to whole genome sequencing. DNA was prepared for sequencing using the Nextera XT DNA Library Preparation Kit (Illumina, Inc.) and sequenced on an Illumina MiSeq. The assembled reads along with the published genome of *R. psychrophilum* were annotated using PROKKA[30] and analyzed in Geneious Prime (v. 2022.2.2) [31]. To identify potential amylolytic enzyme-encoding genes in the genomes of Ika2 and Rho13, a local sequence list containing amino acid sequences of characterized amylases representing all relevant GH groups (GH13, 14, 15, 57, 97, 119, and 126) in the CAZy database was created and blasted against the genome sequence (tblastn) with a max E-value of 10 to include also low-scoring sequence hits. All potential hits were analyzed for sequence-based and *in*

silico structural similarities to known enzymes and enzyme-encoding genes using the Blast module (blastn, blastp, and tblastn) of Geneious Prime against the nucleotide collection (nr/nt) database, Protein Data Bank (pdb), Swiss-Prot, Uni-prot [32,33], and HHPred (MPI Bioinformatics Tools) [34].

The selected “best candidates” were analyzed in InterPro[35] to confirm their classification, and signal peptides were further confirmed by SignalP 6.0[36].

Cloning, expression and purification

Ika2: A gene predicted to encode an enzyme with sequence similarities to α -amylases, neopoullulanases and cyclomaltodextrinases was selected for expression. The gene was amplified using gene-specific primers with overhangs designed for In-Fusion® cloning (Takara Bio Inc.) into plasmid pET28a(+) including a C-terminal 6x His-tag: *Ika*-amy2-F (5'-AGGAGATATACCATGTTATTAGAAGCAATTTACCATCGGC-3') and *Ika*-amy2-R (5'-GTGGTGGTGGTGGTGGGCACCGCTTTTGATGATGAC-3'). Primers used to amplify the vector were pET28a-F (5'-CACCACCACCACCACCACT-3') and pET28a-R (5'-CATGGTATATCTCCTTCTTAAAGTT-3') (vector-specific overhangs are underlined).

DNA fragments were amplified using 2X Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific) with the following PCR protocol: 1 cycle at 98°C for 30 s, 25 cycles at (98°C for 10 s, 55°C for 1 min, 72°C for 30 s/kb), and 1 cycle at 72°C for 5 min (amylase gene)/10 min (vector). PCR products were visualized on a 1% agarose gel and purified using the Monarch® DNA Gel Extraction Kit (New England Biolabs). In-Fusion® cloning was carried out according to the protocol supplied by Takara Bio using 5X In-Fusion Snap Assembly Master Mix (Takara Bio Inc.). In brief, 2 μ l 5X Master Mix was mixed with 50-100 ng purified vector DNA and 50-100 ng purified insert DNA and incubated at 50°C for 15 min followed by incubation on ice for 15 min. The reaction was used directly for transformation into *E. coli* BL21(DE3). Transformed cells were grown on LB agar with 50 μ g/ml kanamycin (Km50) at 37°C overnight.

Rho13 and I3C6: Genes encoding the two putative amylases were codon optimized for *E. coli* and cloned by Genscript (Rijswijk, Netherlands) directly into plasmid pET28a(+) including a C-terminal 6x His-tag. Signal sequences were excluded. Plasmids were transformed into *E. coli* BL21(DE3) for expression. A single colony from each of the three clones was inoculated in 10 ml LB Km50 and incubated overnight at 37°C at 200 rpm shaking. Each overnight culture was used to inoculate 1 L of LB Km50, which was incubated at 37°C at 200 rpm for 4 h. The cultures were subsequently incubated at RT at 200 rpm for 20 min before adding 1 mM isopropyl β -D-1-thiogalactopyranoside for induction. The induced cultures were incubated at 18°C at 200 rpm overnight. Cells from the induced overnight cultures were harvested by centrifugation (10 min, 4500 rpm, 4 °C). The supernatant was discarded, and the pellet was resuspended in 12.5 ml Buffer A (50 mM Tris, 50 mM NaCl, pH 8). Cells were lysed by sonication (5 min., 20% amplitude, pulses of 20 s with 20 s on ice in between). The lysates were centrifuged for 30 min at 15,000 \times g (4 °C), and the supernatant was filtrated through a 0.45 μ m Millipore filter. The filtrate was added to a 15-ml purification column consisting of 4 ml Ni-NTA beads (Thermo Fisher Scientific, Waltham, MA, USA) which had beforehand been charged with 4 ml 1M NiSO₄ and equilibrated with washing buffer (buffer A + 20 mM imidazole). After addition of filtrate, the column was incubated for 15 min, washed with 15 ml wash buffer, and the amylase was eluted with 7 ml elution buffer (Buffer A + 400 mM imidazole).

The eluates were desalted on PD-10 columns (Cytiva, Marlborough, MA, USA) before further purification using an Äkta Pure Protein Purification System (Cytiva) on a Superdex-200 Increase column with a flowrate of 0.5 ml/min. Purifications were carried out in either (1) 100 mM HEPES with 150 mM NaCl and 2 mM CaCl₂ at pH 6.5 or (2) 50 mM MOPS with 50 mM NaCl and 2 mM CaCl₂ at pH 7.5. Fractions giving rise to absorption at 280 nm were examined for activity using an MBTH-assay (see below) and run on SDS-PAGE to assess purity. The purified enzymes were further concentrated using an Amicon ultra 30K spin filter. Mw, pI and extinction coefficients were calculated with the ProtParam tool (<https://web.expasy.org/protparam/>). Protein concentration was measured using Nanodrop ND-1000 (Thermo Fisher Scientific).

Determination of carbohydrate breakdown products

As amylase substrates we used potato starch, beta- and gamma-cyclodextrins, *i.e.* β -CD and γ -CD (Wacher), maltose (reagent grade, VWR) and malto-oligosaccharide preparations of DP3 (maltotriose > 90%), DP6 (maltohexaose > 65 %) and DP7 (maltoheptaose > 60 %) respectively (Merck). For hydrolysis reactions, each enzyme was incubated with 5 g/L starch or CD in 50 mM MOPS buffer pH 7.5 and 2mM CaCl_2 along with either Ika2 (135 nM) and I3C6 (300 nM) at 30°C or Rho (135 nM) at 35°C. Total volume: 1 mL, sampling 90 μL at 0, 2, and 4 h. Due to the low activity of I3C6, it was also sampled at 24 and 48 h. For potential transglycosylation assays, I3C6 was incubated with 11.6 mM glucose/fucose in combination with maltoheptaose and Rho13 with 11.6 mM glucose/fucose in combination with maltohexaose. Ika2 was incubated in 11.6 mM glucose only, 11.6 mM glucose in combination with maltose, maltotriose, maltoheptaose and γ -CD respectively and 11.6 mM fucose in combination with glucose and γ -CD respectively. All reactions were stopped by adding 0.5 M NaOH to the sample 1:1 (v/v). For all assays, controls with only enzyme and no substrate as well as substrate without enzyme were included in the experiments. All samples were analysed by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection on an ICS-6000 system (Dionex™, Thermo Scientific). The system was fitted with a CarboPac PA1 column at 25°C, flow rate: 0.4 mL / min and injection volume: 25 μL . The eluents were A) 1M sodium hydroxide, B) 1M sodium acetate and C) milliQ-water. The eluent contained constantly 10% A) and from 0-5min 1% B) and 89% C); from 5–30 min a linear gradient of B) increasing to 30% at the expense of C), from 30–35 min 90% B) at the expense of C) and from 35–45 min original conditions equilibrating the system for the next analysis. We used a detector gold working electrode; pH-Ag-AgCl reference and a Dionex standard waveform potential cycle for carbohydrates. Standards included glucose, maltose, maltooligosaccharides of DP 3-7, β -CD and γ -CD.

Activity assays

MBTH amylase activity assay: Enzymatic activity was determined by measuring the amount of reducing carbohydrate ends in a 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) assay[37]. For each experiment, 10 mg/ml soluble potato starch (Sigma Aldrich) was dissolved in an assay-specific buffer (see below) followed by acid-catalyzed hydrolysis (adjustment of pH to 1.8 using 6M HCl followed by 45 min at 95°C). pH was then adjusted to the desired assay pH with 6M NaOH, and the concentration reduced to 5mg/ml. The activity assays were performed at 21°C in a reaction mix consisting of 135-200 nM amylase and 4.5mg/ml hydrolyzed starch in the appropriate assay buffer. Every 30 s, 25 μL of the reaction mix was removed and the reaction stopped with 25 μL 0.5 M NaOH. Subsequently, 25 μL MBTH reagent (1.5mg/ml MBTH and 0.5 mg/ml DTT) was added and incubated at 80°C for 15 min. Immediately after removal from the heater, 50 μL developing agent was added (0.5% $(\text{FeNH}_4(\text{SO}_4)_2) \cdot 12\text{H}_2\text{O}$, 0.5% sulfamic acid, 0.25 M HCl, or 0.5M HCl for the pH assay) and samples were left to develop and cool for at least 15 min. After cooling, 20 μL sample was diluted in 180 μL Milli-Q H_2O in a 96 well-plate and absorbance was measured at 620 nm. If absorbance was measured to be higher than the standard curve, samples were further diluted 20-fold in Milli-Q H_2O . Absorbance was converted to reducing ends using a standard curve made from a dilution series of 0.122-62.5 nmol maltose. Activity/initial velocity was determined from linear regression of the increasing number of reducing ends over 330 seconds.

pH optimum: The assay buffer was 100 mM bis-tris propane, 50 mM NaCl, 2 mM CaCl_2 . The starch solution was prepared in assay buffer, and pH was adjusted to pH 6-9.5 (6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5) with NaOH and HCl. Enzyme was mixed with assay buffer of the relevant pH and allowed to incubate for 15 min at RT before starch was added. Initial velocity was measured over 5.5 min. Enzyme concentrations: Rho13: 200 nM, I3C6: 135 nM, Ika2: 136 nM.

NaCl optimum: Assay buffer: 50 mM MOPS, 2 mM CaCl_2 , pH 7.5, NaCl: 0-1000 mM (0, 10, 20, 50, 100, 250, 500, 1000 mM). Substrate and assay buffer were prepared without NaCl, but pH was adjusted with NaOH (given MOPS' pKa of 7.2, the buffer contributes ca. 34 mM to ionic strength). The desired NaCl concentration was achieved using a 2M NaCl stock in assay buffer. Initial velocity was measured over 5.5 min. Enzyme concentrations: Rho13: 200 nM, I3C6: 140 nM, Ika2: 136 nM.

Ca²⁺ optimum: Assay buffer: 50 mM MOPS, 50 mM NaCl, pH 7.5, CaCl₂: 0-20 mM (0, 0.01, 0.05, 0.2, 0.5, 2, 5, 20 mM). Substrate and assay buffer were prepared without CaCl₂ and the desired CaCl₂ concentration was achieved using a 100 mM CaCl₂ stock in assay buffer. Enzymes were buffer exchanged by size exclusion chromatography (SEC) purification on an Äkta pure system on a Superdex-200 Increase column in assay buffer without CaCl₂. Enzyme concentrations: Rho13: 200 nM, I3C6: 135 nM, Ika2: 136 nM. The activity as a function of Ca(II) concentration showed a bell-shaped curve and was fitted to eq. 1[38]:

$$V_{max}^{apo} = \frac{\frac{V_{max}^{apo} \cdot K_{L_1}}{[Ca(II)]} + V_{max}^{L_1} + \frac{V_{max}^{L_2} \cdot [Ca(II)]}{K_{L_2}}}{\frac{K_{L_1}}{[Ca(II)]} + 1 + \frac{[Ca(II)]}{K_{L_2}}} \quad (1)$$

Here V_{max}^{apo} , $V_{max}^{L_1}$ and $V_{max}^{L_2}$ are the maximum velocities of the enzyme with zero, one and two ligand molecules bound, respectively. K_{L_1} and K_{L_2} are dissociation constants for binding of the first and second ligand molecule, respectively. This model is simplified as the proteins might have several binding sites.

Temperature optimum: Assay buffer: e Enzyme was mixed with buffer and placed on ice until assay start. The enzyme and the substrate were individually heated to assay temperature (5, 10, 15, 20, 25, 30, 35, 40, 45, 55°C) for 15 min before the substrate was added to the enzyme solution and the temperature was kept constant during the experiment. Every 30s 25µl of the reaction was stopped in 0.5M NaOH and the activity was measured using the MBTH assay. Activity was determined as a linear fit of the initial 5.5min of the reaction. The enzyme was prepared in 50 mM MOPS buffer pH 7.5 without CaCl₂ but was then diluted 1:4 in assay buffer containing CaCl₂ for 15 min before assay start. Enzyme concentrations: Rho13: 115 nM, I3C6: 50 nM, Ika2: 580 nM.

Biophysical analysis

Circular Dichroism (CiD): The secondary structure of each enzyme (200 µl, 0.2 mg/ml) in 10 mM MOPS, 50 mM NaCl, 2 mM CaCl₂ with or without 3mM EDTA was measured on a Chirascan-plus qCD spectrometer (Applied Photophysics, Surrey, UK). The sample was exposed to a temperature ramp of 1°C/min from 8-90°C while recording spectra at 200-260 nm in 1 nm steps. The bandwidth was set to 1 nm and measurement time to 0.5 s/nm. The recorded temperature was measured directly in the cuvette. Wavelength spectra are shown with buffer background subtracted. Thermal scans were fitted in OriginPro 2024 to following two-transition model (eq. 2) to determine the global melting temperature t_m (indicated in °C).

$$CD_{app}(t) = \frac{(bs_N + as_N * t) * e^{\frac{\Delta H_1}{R * (t+273.15)} * \frac{1 - \frac{t+273.15}{t_{m1}+273.15}}}{e^{\frac{\Delta H_1}{R * (t+273.15)} * \frac{1 - \frac{t+273.15}{t_{m1}+273.15}}}{+ 1 + e^{\frac{-\Delta H_2}{R * (t+273.15)} * \frac{1 - \frac{t+273.15}{t_{m2}+273.15}}}}} + (bs_I + as_I * t) + (bs_D + as_D * t) * e^{\frac{-\Delta H_2}{R * (t+273.15)} * \frac{1 - \frac{t+273.15}{t_{m2}+273.15}}}} \quad (2)$$

With the CiD_{app} signal as a function of temperature and t_{m1} and t_{m2} representing the two melting points and ΔH_1 and ΔH_2 representing the melting enthalpies respectively, R is the gas constant and $bs_N + as_N * t$, $bs_I + as_I * t$, and $bs_D + as_D * t$ the CiD signal for the native, intermediate and denatured species respectively.

Differential scanning fluorimetry: Loss of tertiary structure during heating was monitored with differential scanning fluorimetry on a NanoTemper Prometheus Panta with 7 µl enzyme (0.1 mg/ml) in 50 mM MOPS, 50 mM NaCl, 2 mM CaCl₂. Excitation was at 280 nm and fluorescence emission at 350 nm and 330 nm was measured in parallel at a scan rate of 1°C/min from 15-95°C. Reversibility of unfolding was measured in the same run, by decreasing the temperature back to 15°C at the same scan rate. The 350 nm/330 nm fluorescence ratio was plotted against temperature and fitted to a two-state unfolding equation (eq. 3) in GraphPad Prism 10.0.2.

$$I_{app} = \frac{I_F + I_{FB} * T + (I_U + I_{UB} * T) * e^{\frac{-\Delta H_{unf} * (1 - T/T_m)}{RT}}}{1 + e^{\frac{-\Delta H_{unf} * (1 - T/T_m)}{RT}}} \quad (3)$$

Here, T is the temperature, T_m is the melting temperature, ΔH is the unfolding enthalpy, R is the gas constant. Fluorescence intensity as a function of temperature is described by $I_F + I_{FB} * T$ and $I_U + I_{UB} * T$, for the folded and unfolded species, respectively.

Size exclusion chromatography (SEC): Chromatograms from the SEC purifications were plotted and compared to the elution profiles of 3 standard globular proteins (lysozyme (14.31 kDa), carbonic anhydrase (28.98 kDa and 57.96 kDa), and BSA (66.46 and 132.93 kDa)). The elution volume with the highest absorbance was defined as the elution volume for each peak. The data was plotted as \log_{10} of the MW as a function of elution volume. Estimated MW was calculated from the linear regression on the plot of five peaks from the standard proteins. The MW of the α -amylases was calculated from their sequence, excluding the signal peptide from Rho13 but including the C-terminal His-tag extension LEHHHHHH (Rho13 and I3C6) or HHHHHH (Ika2).

Structure prediction: Alphafold2 was used to predict structure of I3C6 and a dimer structure of Ika2 using the multimer setting used for SAXS [39]. The top result was relaxed with AMBER. Rho13 monomer and dimer structures were predicted using CoLabFold[40].

Small-angle X-ray scattering (SAXS) data collection: All SAXS data were obtained at Aarhus University using an in-house flux-optimized Bruker (Karlsruhe, Germany) SAXS NanoStar instrument with a Ga liquid metal jet source from Excillum (Kista, Sweden) and home-built scatterless slits [41]. All measurements were performed for 1800 s at 25°C. Buffer and noise contributions were measured and subtracted. The resulting data were normalized to absolute scale using scattering from water and logarithmically rebinned. Scattering intensity is described as a function of the modulus of the scattering vector q , $q = (4\pi \sin(\theta))/\lambda$, where 2θ is the scattering angle and $\lambda = 1.34 \text{ \AA}$ is the radiation wavelength for the Ga radiation. SAXS data were acquired using different concentrations for each protein to check for possible concentration effects. All SAXS data treatment and analysis described in the text were done using SUPERSAXS software package (J.S. Pedersen and C.L.P. Oliveira, unpublished). Model-independent analysis was performed by an indirect Fourier transformation routine [42,43] to acquire the pair distance distribution function, $\rho(r)$, from which the maximum distance (D_{max}), forward scattering ($I(0)$), and the radius of gyration (R_g) can be obtained. The value $I(0)$ is used to estimate the molecular weight, M , of the scatterer particle using the equation $M = \frac{I(0)}{c\Delta\rho_m^2}$ where c is the mass concentration per milliliter and $\Delta\rho_m$ is the excess scattering length density per mass, which can be approximated as $2.0 \times 10^{10} \frac{cm}{g}$ for proteins. From this calculated value and the theoretical molecular weight of each monomer, an oligomerization state can be determined.

SAXS data analysis: Model-dependent analysis was performed using structures predicted by AlphaFold in combination with the home written programs *wlsq_symxv6XL*[44] and *wlsq_lin*. The program *wlsq_symxv6XL* calculates theoretical scattering curves of PDB structures with a contribution from a hydration layer by applying the Debye equation using a Gaussian form factor for all non-H atoms. Since the data is normalized to absolute scale, the concentration can be used as a scale parameter and therefore compared with the value obtained from absorbance measurements. Additionally, possible oligomerization states can be optimized from the starting structure using different symmetries, which is more thoroughly described in [45]. This feature was applied to Rho using P2 symmetry, which corresponds to a simple two-fold symmetry. The program *wlsq_lin* is used to fit a linear combination of different oligomer scattering profiles in cases where SAXS model-independent analysis and SEC suggested coexistence of multiple species.

Molecular Dynamics simulations

To assess the overall stability and flexibility of I3C6, Ika2 and Rho13, we conducted Molecular Dynamics (MD) simulations of these enzymes and some close homologs. Prior to the simulations, the predicted structures of the psychrophilic enzymes were aligned with the structures of their

mesophilic/thermophilic counterparts. I3C6 (monomer) was aligned with the α -amylase from *Bacillus stearothermophilus* (PDB: 1HVX[46]; backbone RMSd \approx 1.03 Å), Ika2 (dimer) was aligned with the neopullulanase from *Geobacillus stearothermophilus* (PDB: 1J0H[47]; backbone RMSd \approx 2.14 Å) and Rho13 (monomer) was aligned with the α -amylase from *Halothermothrix orenii* (PDB: 1WZA[48]; backbone RMSd \approx 2.65 Å). After careful analyses of the aligned structures, we copied the coordinates of the $\text{Ca}^{2+}/\text{Na}^{+}$ ions from the homologs' crystallographic structures to the respective psychrophilic structures (**Supplementary Figure S1**). The protonation state of all residues at the optimum pH for each enzyme (see Results) was initially based on the results of the pK_a predictor software PROPKA 3.5.0[49]. Additionally, the chosen protonation states were carefully analyzed and validated through visual inspection of the residues and their surrounding environment.

The ANTECHAMBER module of AMBER 18 [50] was employed to neutralize (with Na^{+} counterions) each amylase and to place it in the center of a rectangular box of TIP3P water molecules[51], whose faces were located at least 15 Å from the surface of each enzyme. All simulations were conducted with the GROMACS 2021.5 software[52] using the AMBER ff14SB force field[53]. Prior to the MD simulation runs, each system was submitted to a multi-step minimization protocol using the steepest descent algorithm[54]. In the first step, both the enzyme and the modeled $\text{Ca}^{2+}/\text{Na}^{+}$ ions were restrained, allowing the minimization of all water molecules; in the second minimization step, the side chains of all residues were relaxed; in the third and last step, the entire system, except the modeled $\text{Ca}^{2+}/\text{Na}^{+}$ ions, was minimized. After the minimization protocol, the obtained systems were used as the starting points of MD simulations, in which the LINCS algorithm[55] was applied to constrain all bonds involving hydrogen atoms. This made it possible to apply an integration time step of 2 fs. In all simulations, the Verlet cut-off scheme was used in combination with a non-bonded cut-off value of 10 Å. Furthermore, periodic boundary conditions were considered, and the Coulomb interactions were treated using the Particle Mesh-Ewald scheme[56]. The minimized systems were first heated for 100 ps to the target temperature (30-35 °C, according to the optimal temperature of each psychrophilic enzyme, see Results) using the V-rescale thermostat [57] in the *NVT* ensemble. During this heating phase, all protein atoms and all modeled $\text{Ca}^{2+}/\text{Na}^{+}$ ions were restrained by a harmonic potential with a force constant of 1000 kJ/mol/nm². Subsequently, a 2 ns equilibration phase was performed in the *NPT* ensemble to relax the system's density to 1.0 bar, accomplished using the Berendsen barostat [58]. The enzymes' backbones and the modeled $\text{Ca}^{2+}/\text{Na}^{+}$ ions were restrained throughout the entire equilibration phase. Finally, a 400 ns production phase was performed, for each system, at constant temperature and pressure (*NPT* ensemble) using the V-rescale thermostat and the Parrinello-Rahman barostat [59]. During this stage, no positional restraints were applied to the system. This three-phased MD protocol was carried out in three replicates, making a total production time of 1.2 μs per amylase system.

To analyse the structural motions of the enzymes, the MD trajectories of the production phase obtained for the three replicates were concatenated to a continuous trajectory of 1.2 μs using the *gmx trjcat* tool. For each system, the root-mean-square deviation (RMSD) of the backbone atoms relative to the initial minimized structure was calculated with the *gmx rms* tool to evaluate the enzyme's stability. The conformational flexibility of the enzymes was evaluated with per-residue root-mean-square-fluctuation (RMSF) analysis, performed on the backbone atoms using the *gmx rmsf* tool. The resulting values provide a comparative measure of psychrophilic versus mesophilic enzyme local and global flexibility. To minimize the strong dependence of the calculated RMSF values on the average structure used as a reference, this particular metric was calculated individually for each replicate and subsequently combined to an average \pm standard deviation value. The compactness of the enzyme structures was assessed by calculating the R_g using the *gmx gyrate* tool. Typically, a higher R_g is suggestive of a less compact and more flexible structure [60]. The solvent-accessible surface area (SASA), calculated with the *gmx sasa* tool, served as another descriptor of structural compactness, with higher SASA values indicating increasing solvent exposure which strongly influences residue flexibility [61]. Principal component analysis (PCA) was employed to reduce the dimensionality of the molecular motions observed in the simulations. For this, the covariance matrices of the atomic

fluctuations of the backbone atoms were constructed and diagonalized using the *gmx covar* tool. Subsequent projection of the trajectory onto the first two eigenvectors was performed using the *gmx anaeig* tool. These eigenvectors represented roughly 50% of the total conformational freedom as evidenced by the cumulative contribution of all eigenvectors. Visualization and image generation of the structural conformations and dynamic trajectories were carried out using VMD 1.9.4 [62] and PyMOL 2.3.0 [63].

3. Results & Discussion

3.1. Isolation, Identification and Recombinant Expression of Amylases

Here we report on the enzymatic and biophysical properties of two new psychrophilic amylases, Rho13 and Ika2, and compare them to our previously reported psychrophilic amylase I3C6 [27]. We start by describing how we identified the two enzymes.

3.1.1. Rho13

A putative GH13-family amylase-coding gene (referred to as Rho13) was identified in the published, PROKKA-annotated genome sequence of the cold-active bacterium *R. psychrophilum* [28,29] identified in ikaite column samples. The gene consisted of 1,545 bp, including a 5'-region encoding a 16 amino acid predicted signal sequence. A BlastP search against PDB showed highest similarity to CAZY GH13 subfamily 36, with the highest similarity to an α -amylase from *Halothermothrix orenii* (pdb: 1WZA) (42.80%) and an amylase from *Eubacterium rectale* (pdb: 7JJN) (33.53%). The sequence of the mature sequence excluding the signal sequence (1,497 bp, i.e. 499 residues) was cloned into a pET28 vector with a C-terminal His-tag and recombinantly expressed in *E. coli* BL21(DE3) with a C-terminal His-tag. This allowed us to purify Rho13 on Ni-NTA columns followed by size exclusion chromatography (SEC), leading to single-band purity (**Figure 1a**).

3.1.2. Ika2

Bacteria-containing material was extracted from ikaite columns in the Ikka Fjord, Greenland, and incubated for three months at 5°C on diluted, R2-based substrate containing AZCL-amylose. An amylase-positive bacterial strain was isolated in this way. To identify the gene encoding the active amylase, the bacterial isolate was subjected to full genome sequencing [31], which allowed us to assign the strain to a novel taxon within the *Bacillaceae* related to *Anaerobacillus*, *Paenibacillus*, and *Alkalihalobacillus*. The PROKKA-annotated genome was mined for putative amylase-encoding genes, and an open reading frame consisting of 1,737 bp was identified as a gene potentially encoding a cyclomaltodextrinase, α -amylase, α -glucosidase or a neopullulanase. In a BlastP search against PDB, the translated gene sequence shared highest similarity to four starch-degrading enzymes belonging to the GH13 subfamily 20 (GH13_20), namely a cyclomaltodextrinase (which linearizes cyclodextrins) from *Bacillus* sp. (pdb: 1EA9) (56.61%) [13], a maltogenic amylase (which releases maltose from starch) from *Thermus* sp. (pdb: 1GVI) (55.2%) [64], a neopullulanase (which hydrolyzes α -1,4 linkages in pullulan through transglycosylation) from *Geobacillus stearothermophilus* (pdb: 1J0H, 1J0J) (53.9%) [47] and α -amylase II (which cleaves starch into smaller oligosaccharides) from *Thermoactinomyces vulgaris* (pdb: 1WZM) (46.5%) [65–67]. The sequence similarity is rather low, but starch-degrading enzymes are known to be a highly diverse group [68], and several of them exhibit multiple catalytic activities. For example, maltogenic amylases hydrolyze both α -D-(1,4)- and α -D-(1,6)-glycosidic bonds and exhibit transglycosylation activation on a range of mono- and disaccharide acceptors [64,69]. *In silico* folding of the putative protein sequence using HHpred showed that it was indeed able to fold into an amylase 3D structure. To verify the amylolytic activity of the identified putative GH13 enzyme (denoted Ika2), the predicted gene was recombinantly expressed with a C-terminal His-tag and purified from *E. coli* in a manner similar to Rho13 (**Figure 1b**).

3.2. Structural and Biophysical Analysis of the Amylases

In the following, we report on the enzymatic activity of the two amylases as a function of temperature, pH, CaCl₂ and NaCl as well as presenting a structural and biophysical analysis *in vitro* and *in silico*. We compare them to the psychrophilic amylase I3C6, which was isolated from a metagenomic analysis of ikaite columns and was expressed and purified recombinantly (**Figure 1c**).

3.2.1. Substrate Specificity and Cleavage Products

We first investigated which carbohydrate substrates were processed and what products were formed by the three enzymes, guided by the activities of the enzymes they had been found to be related to (see previous section). To measure hydrolytic activity, the amylases were incubated with starch or cyclodextrins (β -CD and γ -CD) and the products analysed by pulsed amperometric detection (PAD) and peaks assigned as described [70]. In addition, we checked for potential transglycosylation activity by incubating the enzymes with either glucose or fucose (a deoxy sugar in the L-conformation) in the presence of a maltooligosaccharide preparation containing maltoheptaose (dp7 where dp is degree of polymerization) or maltohexaose (dp6). For a better overview, representative profiles are shown in **Figure 2a-c**, while additional PAD elution profiles are shown in **Figure S2a-h**.

Ika2

After 4 h of incubation, Ika2 hydrolyzed starch to glucose, maltose and a series of maltodextrins with increasing dp (**Figure 2a**). In the same period, β -CD was hydrolysed to glucose, maltose, maltotriose as well as smaller amounts of maltodextrins up to dp7 and a product tentatively assigned to isomaltose (**Figure S2a**). A similar pattern was observed with γ -CD (**Figure 2b**), which was degraded to glucose, maltose and maltodextrins up to dp8; maltooctaose (corresponding to linearized γ -CD) was degraded further after 4 h incubation. Incubation with fucose and the maltooligosaccharide preparation containing maltoheptaose showed that fucose remained unconverted, while glucose and maltose were formed at the expense of maltooligomers (maltohexaose and -heptaose in particular) (**Figure 2c**). In addition, products appeared tentatively assigned to isomaltose. Products eluting with retention times between maltose and dp5 are presumably positional isomers of dp3-dp5 and remained after 48 h of incubation, suggesting transglycosylation activity (see arrows in **Figure 2c**). The degradation of cyclodextrins points to endo activity, and the degradation of starch and malto-oligomers resulted in the formation of glucose as one of the products.

Rho13

Starch was hydrolysed and formation of glucose, maltose and maltotriose was observed, with further degradation of the latter resulting in glucose and maltose as the main products after 4 h of incubation. Unlike Ika2 (and I3C6, see below), maltodextrins with dp > 6 seemed absent. β -CD was degraded to glucose, maltose and maltotriose as well as small amounts of maltotetraose (**Figure S2b**). γ -CD was degraded to glucose, maltose and malto-oligosaccharides of DP 3-7 after 4 h of incubation (**Figure S2c**), as well as positional isomers of maltose (isomaltose), maltotriose and maltotetraose, though the distribution of dp3-4 isomers differed subtly from that of Ika2. Overall γ -CD seemed a better substrate than β -CD, given the larger concentrations of products. Incubation with glucose and a maltooligosaccharide preparation primarily containing DP 4-6 showed only a small increase in glucose concentration but a significant production of maltose with degradation of maltooligomers already after 4 hrs, with maltotriose being produced after 4 hrs but degraded after 48 hrs (**Figure S2d**). Rho13's degradation of cyclodextrins also indicates endo-activity, like Ika2, though the ability to degrade maltotriose and maltotetraose illustrates a degree of exo-activity as well.

I3C6

No activity of the enzyme was observed on cyclodextrins when compared with the zero-time sample (**Figure S2ef**). There was a lower level of starch degradation than with Ika2, consistent with the low general activity of I3C6. When glucose was co-incubated with maltoheptaose, a dramatic reduction in glucose was observed with a simultaneous increase in maltose, but there was no detectable change in maltoheptaose (**Figure S2g**). While combined incubation with fucose and maltoheptaose showed no significant change in fucose (eluting around 2 min), maltoheptaose was degraded to smaller maltooligomers including maltose but not glucose (**Figure S2h**). Given I3C6's modest activity on starch and the lack of degradation of the cyclodextrins, our investigation suggests that this enzyme has little endo-activity but a preference for degradation of maltoheptaose in the absence of glucose.

In summary, the three enzymes showed distinct profiles towards different carbohydrate substrates, with variable levels of hydrolytic activity towards starch, cyclodextrins and maltooligosaccharides, and only Ika2 showing evidence for transglycosylation activity.

3.3. Ika2 Forms a Stable Dimer While Rho13 and I3C6 Are Mainly Monomeric

The three enzymes showed different elution behaviour on a size exclusion column (**Figure 1**). Rho13 eluted with two major peaks, both of which corresponded to the pure protein according to SDS-PAGE. Both peaks could be fitted to single Gaussian curves (**Figure 1a**). Based on calibration of the column with proteins of known size, the first peak had a retention volume corresponding to a dimer and the second that of a monomer (**Figure S3**). The dimer and monomer peaks correspond to 26 and 74% of the combined peak area respectively, and both peaks showed significant enzymatic activity (based on measuring the formation of reducing ends in an MBTH-assay[71]). Ika2 eluted as a single peak, though this was slightly skewed (**Figure 1b**). The observed peak top around 13 mL predicted a molecular weight of 92 kDa, lying between the monomeric and dimeric values of 69 and 138 kDa, respectively. This suggests a dynamic equilibrium between monomer and dimer which exchange too rapidly to elute as separate peaks. I3C6 also showed a major peak preceded by a broad but low plateau (**Figure 1c**). Only the major peak (which corresponded to 84% of the total elution area) showed significant activity. The peaks eluting prior to the main I3C6 peak were smaller, but bands corresponding to monomeric I3C6 were reproducibly observed by SDS-PAGE in the corresponding lanes (**Figure 1c**), and we therefore attribute them to unspecific aggregates or oligomers of I3C6 which dissociate during SDS-PAGE.

Table 1. Oligomerization properties of the 3 enzymes probed by SAXS.

Protein	Concentration _{Abs280} (mg/mL)	R _g (Å) ^a	Model	χ ² ^b	Concentration _{SAXS} (mg/mL)	n-mers ^a (SAXS ^b)
Ika2	1.99	33.8 ± 0.1	AF dimer	4.3	1.69	1.67 (1.97)
	1.00	33.8 ± 0.1	AF dimer	1.9	0.84	1.67 (1.99)
	0.51	34 ± 0.2	AF dimer	1.4	0.42	1.63 (1.96)
Rho	0.94	49.8 ± 0.5	Monomer _{0.03 mg/mL} +Dimer _{0.69 mg/mL}	2.9	0.72	1.58 (1.96)
	0.46	42.6 ± 0.7	Monomer _{0.06 mg/mL} +Dimer _{0.29 mg/mL}	1.4	0.35	1.38 (1.83)

I3C6	1.76	64.5 ± 1	-	-	-	1.6
	0.87	94 ± 0.8	-	-	-	3.42
	0.46	111.4 ± 0.4	-	-	-	7.4

Notes: a. Determined from the indirect Fourier transformation routine as described in the method section. b. SAXS modelling results using the described model. Protein concentrations obtained from the model fit using absolute scale.

To elucidate the oligomerization states of the proteins more directly, we recorded SAXS curves of the three enzymes (**Figure 3a, Table 1**). For I3C6, the radius of gyration (R_g) increased with protein concentration (**Figure 3b**). We attribute this to non-specific aggregation, since the SAXS data could not be fitted to a mix of monomer and dimer. In contrast, Rho13 data was best fit by a combination of the AlphaFold-predicted structure for the monomer and the symmetry-built dimer. Furthermore, all concentrations could be described using the linear combinations of the models with some deviation at higher concentrations, suggesting a dimerization equilibrium of Rho13 (**Figure 3b**) as expected from SEC data. SAXS curves of Ika2 were fitted using an AlphaFold-predicted Ika2 dimer. This led to a value of R_g independent of concentration and consistent with the R_g calculated for a dimer. Dimerization is a common feature for many of Ika2's closest homologs (54-57% identity): amylase from *Thermus* sp. (PDB: 1SMA) is a dimer [64] and cyclomaltodextrinase from *Bacillus* sp. (PDB: 1EA9) is a dodecameric hexamer of dimers [13], and neopullulanase from *Geobacillus stearothermophilus* (PDB: 1J0H) is also a dimer [47]. In addition, 8 of the 10 published structures in GH13_20 form dimers.

3.4. Activity Profiles as a Function of Temperature, pH and Ca^{2+}

Enzymatic activity (hydrolysis of starch) under a range of different conditions was determined using the MBTH assay, which measures formation of reducing carbohydrate ends. The organisms from which the three enzymes are obtained all live in a cold environment, that is a mixture of seawater and alkaline spring water, making it reasonable to expect that they would prefer an alkaline and modestly saline environment with a low optimal temperature. We therefore examined the impact of temperature, pH and salt.

3.4.1. Temperature

All three enzymes were preincubated at a given temperature for 15 minutes, after which product formation was measured from a linear increase in product signal over a 5.5-minute period (see typical examples in **Figure S4a**). Measured in terms of specific activity (moles reducing ends formed per minute per mole enzyme) at pH 7.5, Rho13 is an order of magnitude more active than the two other enzymes (**Figure 4a**), on average 30- and 15-fold more active than Ika2 and I3C6, respectively. However, when normalized to each enzyme's maximal activity, the thermal profiles of the three amylases were similar, with optima of 30-35°C (**Figure 4b**) and significant activity below 20°C. Ika2 showed particularly pronounced psychrophilic properties with 50% of maximal activity at 5°C, as well as being the most thermostable, with 40% activity remaining at 55°C. However, all three enzymes can be classified as psychrophiles, with significant activity below 20°C.

3.4.2. pH Profile

Rho13 and I3C6 showed a clear preference for higher pH, with optimal activity around pH 8-9. However, they differed in their alkaline sensitivity: I3C6 was completely inactive already at pH 9.5, while Rho13 still showed 78% activity at this pH. In contrast, Ika2 was markedly acidophilic (despite its origin in Ikaite columns) with a marked decline in activity from pH 6 up to pH 8 (**Figure 4c**). At

pH 6.0, Ika2 activity has increased ca. 3.3-fold compared to pH 7.5 while that of Rho13 has declined ca. 7.5-fold, making Ika2 ~1.5-fold more active than Rho13 at pH 6, while the absolute activity of Rho13 at its optimal pH of 8.0 is still at least 7-fold higher than that of Ika2 at pH 6. Thus, Rho13 is overall significantly more active than the two other enzymes.

3.4.3. Salt Profile

All 3 enzymes are reasonably halotolerant, though Ika2 and I3C6 show some decline in activity > 150 mM NaCl (**Figure 4d**).

In summary, the three enzymes all showed significant activity at low temperatures, peaking slightly above ambient temperatures, but showed individual variations in terms of sensitivity to pH and ionic strength. Ika2 has the broadest temperature profile and only moderate dependence on salt, but its preference for low pH makes it less appealing as a detergent enzyme, given the preference for alkaline conditions in *e.g.* laundry washing [21]. Here Rho13 has the advantage of a strongly alkaliphilic profile as well as a robust indifference to ionic strength, though its activity declines more steeply with lowered temperature than Ika2. I3C6 has the weakest attributes with a low specific activity, complete abolition of activity > pH 9.0 and a slow decline in activity with ionic strength. As a final differentiator of activity, we turn to the impact of Ca^{2+} on enzyme activity.

3.4.4. Analysis of Ca^{2+} -Binding of Ika2, Rho13 and I3C6

As we will describe in detail below, there is good reason to expect Ca^{2+} to bind to all three enzymes and affect activity and stability. Accordingly, in the following section we describe the experimental effect of Ca^{2+} ions on the activity, structure and stability of the 3 enzymes and then examine the structural basis for the role of Ca^{2+} based on computational analysis. Activity was measured in the MBTH assay (**Figure 4e**), structure by far-UV circular dichroism (CiD, **Figure 5a**), while thermal stability was determined either through changes in the proteins' secondary structure by CiD (**Figure 5b**) or tertiary structure by differential scanning fluorimetry (DSF) (**Figure 5c**). CiD data could in all cases be fitted with a 3-state model, involving the native state N, a partially denatured intermediate I and a denatured state D. Where possible, data from 21 different wavelengths (210-230nm in steps of 1 nm) or 9 different wavelengths (Ika2 with no EDTA) were fitted globally to obtain the melting temperatures for the two transitions N-to-I and I-to-D. Results are summarized in **Table 2** and described in detail below.

Ca^{2+} Has Little Effect on Ika2 Activity but Rho13 and I3C6 Follow a Bell-Shaped Dependence

Ika2 showed no increase in activity upon addition of Ca^{2+} up to 20 mM, but rather a slight decline (**Figure 4e**). For this reason, activity was determined in the presence of EDTA, which had no significant influence on activity, suggesting that Ca^{2+} has no positive effect on activity (**Figure S4b**, time profiles in **Figure S4c**). In contrast to Ika2, addition of Ca^{2+} to both Rho13 and I3C6 shows a bell-shaped curve with an activity optimum around 2.5 mM CaCl_2 (**Figure 4e**). The positive effect of Ca^{2+} on activity can be seen directly in the time profiles when adding either EDTA or CaCl_2 at different time points (**Figure S4c**). The activity data can formally be fitted to a model in which the first somewhat weak binding step (inflection point ca. 0.5 mM) increases activity and the second, even weaker, binding step (inflection point ca. 8-20 mM) decreases it. The decline in activity at higher Ca^{2+} concentrations is indicative of unspecific inhibition, seen for numerous other enzymes. In the case of Rho13, we speculate that this second binding event might involve consensus sequence 2 (CS2, see below), since a Ca^{2+} binding here would increase rigidity and lower activity.

Removal of Ca^{2+} Affects Enzyme Structure and Reduces Stability

Rho13

Thermal denaturation by far-UV CiD in the absence of EDTA showed a gradual redshift from a 222 nm peak to a flatter curve from around 53 °C onwards (**Figure 5b**). The shift was fitted in OriginPro2024 to a three-state unfolding model, with the first transition corresponding to a global denaturation and the second interpreted to unspecific aggregation. This first unfolding step showed a t_m of $55.98 \pm 0.04^\circ\text{C}$ and the second $58.32 \pm 0.14^\circ\text{C}$. Consistent with this, DSF showed a transition of $55.215 \pm 0.007^\circ\text{C}$, with no reversibility of unfolding (**Figure S5**). The t_m was significantly above the temperature optimum of 35°C (**Figure 4b**). Activity declines steeply above 45°C . This suggests a local unfolding of the enzyme that presumably involves the active site, thus leading to thermal inactivation of Rho13 observed in the MBTH assays. There was no significant change in far-UV CiD structure of Rho13 with and without EDTA (**Figure 5a**), but the enzyme showed a significantly changed CiD unfolding curve (**Figure 5b**), suggesting the existence of a structurally important Ca^{2+} binding site. Consistent with this, DSF shows that EDTA lowers the t_m by 2°C .

Ika2

While EDTA did not affect Ika2’s activity (**Figure 4e** and **Figure S4bc**), it significantly altered the enzyme’s secondary structure according to far-UV CiD (**Figure 5a**). The major minimum around 208 nm was redshifted, and intensity was amplified. This suggests a Ca^{2+} binding site, which is structurally important as seen in the effect on stability, but with a deleterious effect on activity above 1 mM CaCl_2 (**Figure 4e**). The thermal denaturation data could not be fitted with great confidence due to visible aggregation at these high temperatures. Nevertheless, an acceptable fit was made predicting two consecutive t_m values of 57.2°C and 62.1°C in 2 mM CaCl_2 (**Table 2**), which change to 47.8°C and 72.2°C in EDTA; the second transition is likely aggregation. DSF, which monitors changes in Trp fluorescence and thus reports on the global structure, shows a single melting transition with a midpoint of 62.0°C which falls dramatically to 50.8°C in EDTA (**Figure 5b** and **Table 2**). Thus, in CaCl_2 the secondary and tertiary structure are both lost around $57\text{--}62^\circ\text{C}$, well above the optimal activity temperature of 30°C .

Table 2. Optimal activity temperatures and melting temperatures t_m obtained from the thermal unfolding of the 3 enzymes Ika2, Rho13 and I3C6 monitored by either DSF or far-UV CiD in the presence of 2 mM CaCl_2 alone or with 3 mM EDTA ^a.

	Activity optimum (°C)	t_m (DSF) (°C) ^b		t_m (CiD) (°C) ^c			
		2mM CaCl_2	2mM CaCl_2 + 3mM EDTA	2mM CaCl_2		2mM CaCl_2 + 3mM EDTA	
		t_m	t_m	$t_m^{\text{N-I}}$	$t_m^{\text{I-D}}$	$t_m^{\text{N-I}}$	$t_m^{\text{I-D}}$
Ika2 ^d	35	61.97 ± 0.01	50.82 ± 0.01	57.16 ± 1.77	62.10 ± 0.99	47.80 ± 0.23	72.16 ± 0.20
Rho13 ^e	30	55.21 ± 0.01	53.38 ± 0.01	55.98 ± 0.04	58.32 ± 0.14	N/A ^g	N/A ^g
I3C6 ^f	30	55.40 ± 0.01	52.28 ± 0.01	52.47 ± 0.05	53.85 ± 0.52	55.67 ± 0.25	75.42 ± 0.70

Notes: ^a Buffer used was 10 mM MOPS pH 7.5, 50 mM NaCl (CiD) or 50 mM MOPS pH 7.5, 50 mM NaCl (DSF). ^b DSF unfolding curves were analyzed as global fits of three technical replicates of 330nm/350nm fluorescence and followed a single transition in which the signal of both the native and the denatured state changed linearly with rising temperature. ^c CiD was measured as measured ellipticity of 210-230nm and showed a three-state

unfolding NàlâD typically with quite narrow intervals of unfolding. The three states were individually fitted as described below with global fits to 21 different wavelengths from 210-230nm (or from 9 different wavelengths from 214-230 for Ika2_{CaCl2}). ^d CiD data for unfolding of Ika2_{CaCl2} did not converge on a fit with the full 214-230nm spectrum, but could be fitted with every second wavelength to a satisfying fit using linear baseline correction of the intermediate state. Ika2_{EDTA} was required baseline correction of all three states. ^e Rho13_{CaCl2} was baseline corrected to a linear change in signal with temperature for native and denatured state while Rho13_{EDTA} showed a completely different unfolding curve, likely suggesting denaturation upon the stripping of the Ca²⁺. ^f I3C6_{CaCl2} required baseline correction of all three states, while I3C6_{EDTA} did not require baseline correction of the native state. ^g Not fitted because data did not show a clear transition.

I3C6

Thermal unfolding of I3C6 measured by CiD and DSF showed t_m values of 52.47°C (first transition) and 55.40 °C, respectively (**Figure 5bc**). EDTA had a small effect on the far-UV CiD spectrum which is likely due to concentration differences and does not show any spectral shifts (**Figure 5a**). Surprisingly, the CiD-measured t_m increased by 3°C to 55.7 ± 0.25°C upon addition of EDTA. This is likely not significant, as the DSF-based t_m values with and without EDTA are reversed compared to CiD (**Table 2**).

In summary, the three enzymes differ significantly in their dependence on Ca²⁺ for stability and activity, and the two phenomena are not correlated. Ika2's activity is unaffected by Ca²⁺, but its removal markedly destabilizes the enzyme; conversely, I3C6 and Rho13 both show optimal activity around 2.5 mM Ca²⁺, but are destabilized to very different extents. Clearly, they must differ considerably in their interactions with Ca²⁺.

3.5. Computational Analysis of Ca²⁺ Binding to the Three Enzymes

Having shown that Ca²⁺ has a profound but complex effect on the three amylases, we now turn to computational analysis of the potential interactions involved.

3.5.1. Ika2 Shows No Evidence for Stable Ca²⁺ Binding

GH13_20 enzymes such as Ika2 often have a metal binding site. Five PDB crystal structures from this family show a bound Ca²⁺ and share high sequence preservation in this region (**Figure 6a**) which involves the amino-acid motif NGDPSND...GGD (with metal ion coordination by the Asp residues). The two closest homologs of Ika2 (1GVI and 1EA9) have no assigned Ca²⁺ in their crystal structure (possibly due to loss during purification and crystallization), though they retain this Ca²⁺ binding motif. We note that the Ca²⁺ binding site is not completely conserved in the GH13_20 family. Thus, 2Z1K, 2WC7 and 4AEF have mutations among the coordinating residues; in addition, in 4AEF the sequence Q224-Y233 is not modelled, indicating a lack of metal coordination. Given Ika2's almost complete conservation of the Ca²⁺ binding motif (**Figure 6a**), it would be expected to bind Ca²⁺; nevertheless, Ca²⁺ has hardly any effect on Ika2 activity (**Figure 4e**). MD simulations corroborate these observations. They show only an increase in the average RMSD and RMSF values of 0.2 Å and 0.1 Å, respectively, in 1.2 µs trajectories conducted with and without Ca²⁺. This suggests that the absence of Ca²⁺ at best slightly increases the flexibility of Ika2. As summarized in **Figure S6**, a comparative analysis of the per-residue RMSF values revealed that only 24 residues, representing 2% of the dimer, exhibited increased fluctuations above the threshold of 0.5 Å in the trajectory without Ca²⁺. Moreover, half of those residues are located at or near the Ca²⁺-binding sites and only five are located near the active site cleft, indicating that the absence of Ca²⁺ should not have a major impact on residues involved in substrate interactions. This further supports our experimental finding that the absence of Ca²⁺ does not have a negative impact on catalytic activity (**Figure 4e**). Similar observations were noted in the Ika2 homolog neopullulanase (PDB code 1J0H), where Ca²⁺ is proposed to be non-essential for catalysis [47].

3.5.2. Rho13 Likely Has 1 Ca²⁺ Binding Site

Solved structures of homologs of Rho13 in the GH family 13_36 have varying numbers of preserved Ca²⁺ binding sites. To the best of our knowledge, Rho13 has two consensus sequences (CS): CS1 (DSDGDGTGD) (**Figure 6b**) and CS2 (VINH---PDL---(HI)/(YF)), identified in 3K8K, 7JJT, and 5M99, at the interface of three loops close to the active site. The α -amylase from *Halothermothrix orenii* (PDB: 1WZA), Rho13's closest homolog, shares these two consensus sequences, but only CS1 is occupied by Ca²⁺. A second Ca²⁺ in 1WZA is lodged in a second Ca²⁺ binding loop (residues 65-77), a structural element also observed in 7JJT, but that binding loop does not exist in Rho13's structure. Therefore, our computational structure of Rho13, used in subsequent MD simulations, contains a single Ca²⁺ ion (**Figure S1a**), which was transferred from the 1WZA homolog to the first conserved Ca²⁺ binding site of Rho13 after a structural alignment of both structures.

3.5.3. I3C6 Only Maintains 1 Ca²⁺ Ion Stably Bound

I3C6 was previously reported to have increased activity when CaCl₂ is added [27], and several I3C6 homologs in the GH family 13_5 have been shown to contain multiple binding sites. The amylase from *Bacillus stearothermophilus* (PDB: 1HVX) has a generally preserved Ca²⁺(I)-Na⁺-Ca²⁺(II) binding site with three ions in line, and a third Ca²⁺(III) binding site located at the interface of two structural domains. Accordingly, we computationally transferred the Ca²⁺(I)-Na⁺-Ca²⁺(II) triad and the Ca²⁺ (III) ion from the 1HVX homolog to the I3C6 structure predicted by AlphaFold (**Figure S7a**) and conducted MD simulations to better understand the Ca²⁺-binding of this psychrophilic enzyme. Interestingly, our simulations revealed that only Ca²⁺ (I) and Na⁺ ions remained in their binding site, whereas Ca²⁺(II) and Ca²⁺(III) abandoned their original positions throughout the simulations (**Figure S7b**). This may have resulted from existing differences in the amino acid composition of the Ca²⁺ binding sites between the psychrophilic enzyme and its homolog. While the Ca²⁺(I)-Na⁺-Ca²⁺(II) binding site of 1HVX exhibits seven Asp residues (D105, D162, D186, D197, D203, D205, and D207), the same region of I3C6 only presents three (D195, D201, and D203) (**Figure S8a**). Consequently, the number of ionic interactions established between the ions and the residues of the binding site is much smaller in I3C6, resulting in a looser binding of the Ca²⁺(I)-Na⁺-Ca²⁺(II) triad. Among the three cations, Ca²⁺(II) established fewest interactions with I3C6 and was most exposed to the solvent, thus explaining its rapid release from the binding site in all simulation attempts. This may also explain the escape of Ca²⁺(III) from its initial position in the MD simulations conducted with I3C6. The region surrounding the transferred Ca²⁺(III) ion contains fewer Asp residues (one in I3C6 *vs.* two in 1HVX) and includes two positive amino acids, K405 and R429, that correspond to S406 and G431 in the 1HVX homolog, respectively (**Figure S8b**). The increase in the positive character of this region makes it less likely to accommodate the Ca²⁺(III) which, without good binding contacts, easily leaves the binding site. In sum, the simulations suggest that only a single Ca²⁺ can persistently bind the I3C6 structure, and this was used for all subsequent MD runs performed with this psychrophilic enzyme.

3.6. Psychrophilic Features of the Three Amylases Analyzed by MD Simulations

Psychrophilic enzymes exhibit distinct structural and dynamical features that allow them to operate effectively in cold environments [2]. Compared to their mesophilic homologs, psychrophilic enzymes are characterized by increased structural flexibility, which is often linked to a lower number of Pro residues, fewer ionic and polar interactions, an increased presence of Gly residues, and enhanced solvent accessibility [72]. Collectively, these factors reduce the energy barriers for molecular movement at lower temperatures and influence activation free energy by respectively decreasing and increasing enthalpic and entropic contributions. According to transition state theory, this adjustment is advantageous, as enthalpy-driven reactions decrease exponentially with temperature reduction, while entropic contributions remain unaffected by temperature changes [73,74]. Nonetheless, excessive flexibility in regions unrelated to catalysis will not alter the enthalpy term but will elevate the entropic barrier. This can be mitigated either by increasing local rigidity in

non-critical regions that do not affect catalytic movements or by concentrating enhanced flexibility in areas relevant to catalysis (albeit not necessarily at the active site) [73,74].

Table 3. Average values \pm standard error of the mean of multiple structural metrics determined for the continuous 1.2 μ s MD trajectories (i.e., three 400 ns-long replicas concatenated into a single trajectory) performed with the three psychrophilic α -amylases (Rho13, Ika2, and I3C6) and their respective homologs (1WZA, 1J0H, and 1HVX).

	Average RMSD (backbone) / Å	Average RMSF (backbone) / Å	Average No. H-bonds	Average R_g (backbone) / Å	Average SASA / nm ²
Rho13	2.763 \pm 0.006	1.02 \pm 0.02	413.81 \pm 0.09	23.46 \pm 0.02	216.56 \pm 0.03
1WZA (homolog)	2.600 \pm 0.004	0.88 \pm 0.02	417.01 \pm 0.09	22.88 \pm 0.02	208.68 \pm 0.03
Ika2	2.837 \pm 0.005	1.295 \pm 0.008	1000.6 \pm 0.1	33.400 \pm 0.002	462.69 \pm 0.06
1J0H (homolog)	2.123 \pm 0.004	1.075 \pm 0.008	999.8 \pm 0.1	33.467 \pm 0.002	458.63 \pm 0.06
I3C6	1.670 \pm 0.003	0.92 \pm 0.02	410.79 \pm 0.09	24.11 \pm 0.02	205.81 \pm 0.03
1HVX (homolog)	1.073 \pm 0.002	0.72 \pm 0.01	424.97 \pm 0.09	23.73 \pm 0.02	186.45 \pm 0.02

To better understand the conformational dynamics of the Rho13, Ika2, and I3C6 psychrophilic amylases, we conducted MD simulations with these three enzymes and with their respective thermophilic homologs (1WZA, 1HVX and 1J0H). We evaluated the RMSD of backbone atoms along the concatenated 1.2 μ s trajectory for the six amylases, using the minimized structure of each enzyme as a reference. The average RMSD values for the psychrophilic enzymes consistently exceeded those observed for the respective mesophilic counterparts (**Table 3**), indicating that the cold-active enzymes are less stable than their corresponding homologs. This aligns with what is hypothesized in the literature [72,75]. Specifically, the I3C6 vs. 1HVX pair showed the largest variance (0.6 Å), while Rho13 vs. 1WZA displayed the smallest difference (0.2 Å). Additionally, the standard deviation of the presented RMSD values was consistently higher in the psychrophilic enzymes, suggesting more pronounced structural fluctuations.

We made a structural comparison of the psychrophilic enzymes and their mesophilic homologs by evaluating the R_g , SASA, and intra-protein hydrogen bond count throughout the concatenated 1.2 μ s simulations. Our findings revealed a lower average number of intra-protein hydrogen bonds and an increased average R_g value for Rho13 and I3C6 (**Table 3**), suggesting a weakening of stabilizing interactions and overall less compact structures compared to their mesophilic counterparts. In contrast, Ika2 showed similar values for hydrogen bonds and R_g relative to its homolog, potentially attributable to its dimeric nature, which may influence these metrics. Nonetheless, Ika2 exhibited a 10 % decrease in the average number of salt bridges compared to its mesophilic counterpart, which also points to a disruption of stabilizing interactions. All three psychrophilic enzymes demonstrated higher average SASA values (**Table 3**), underscoring a trend toward decreased compactness and improved interactions with the solvent.

RMSF values (i.e. levels of structural changes during MD simulations) for the three psychrophilic enzymes and their mesophilic counterparts reinforced our findings on psychrophilic conformational dynamics (**Figure S9**). The RMSF values of the backbone consistently indicated an overall increase in the structural flexibility of psychrophilic enzymes compared to the mesophilic variants (**Table 3**). However, drawing significant conclusions from average RMSF values can be challenging in view of the substantial standard deviation values that were observed. To gain deeper insights, we delved into residue-specific flexibility by comparing the RMSF values of corresponding residues between each psychrophilic enzyme and its mesophilic counterpart, using a $\pm 0.5\text{\AA}$ threshold to identify notable changes in the flexibility of each residue. The cold-adapted amylases exhibited a larger number of residues exceeding this threshold (**Figure 7a-c**, represented in magenta sticks), indicating that larger portions of the psychrophilic enzymes have increased flexibility relative to their mesophilic homologs.

Ika2 displayed 111 residues with at least 0.5Å increase in RMSF values compared to their corresponding residues in the 1J0H homolog (**Figure 7b**), constituting approximately 10% of the enzyme, which indicates an enhanced global flexibility. Conversely, 16 residues exhibited > 0.5Å decrease in flexibility, indicating targeted increases in rigidity. Interestingly, the flexibility distribution varied between the two chains of the enzyme. Among the residues with increased flexibility, 76 were located on chain A and 35 on chain B, whereas increased rigidity was more pronounced in chain B with 13 residues, compared to only three in chain A. A domain-specific analysis revealed that 40% of the residues with increased flexibility are located within domain N (**Figure 7b**), which is crucial for defining the active site cleft and is known to undergo substantial structural rearrangements upon substrate binding in the homolog neopullulanase [47]. This suggests a potential role for this domain in enhancing substrate interaction at low temperatures. Domain A, which contains the common (β/α)₈-barrel motif found in amylase family enzymes and together with domain N forms the active site cleft, accounted for 32% of the residues with increased flexibility (**Figure 7b**). Conversely, domain B had the lowest percentage of residues with increased flexibility (2%) and the highest percentage (56%) of residues with increased rigidity (*i.e.*, residues whose RMSF is > 0.5 Å below the RMSF of the equivalent residues of the mesophilic homolog enzyme—**Figure 7b**, represented in yellow sticks), underscoring its potential role in maintaining structural integrity near the catalytic site. Domain C, which is the farthest from the active site and features solvent-exposed mobile loops, had 27 % of the residues with increased flexibility. Of note, 17 of the 111 residues that showed increased flexibility are in important positions for substrate recognition and interaction, as identified in substrate-enzyme complex structures of the homolog (PDB codes 1J0I, 1J0J, and 1J0K). Notably, none of the catalytic residues in Ika2, *i.e.* the critical D330/E359/D426 triad and others promoting relevant ionic and polar interactions for catalysis (Y209, H249, R328, H425, D470, and R474) showed increased flexibility, in accord with the literature [72,74].

Rho13 and I3C6 have 21 and 30 residues, respectively, with increased flexibility compared to the matching 1WZA and 1HVX homologs (**Figure 7a and 6c**, represented in magenta sticks). Most of these residues are in domain A (nearly 57% of all residues with increased flexibility), specifically in the region that forms the active site cleft, as also observed for the Ika2 amylase. We believe that the enhanced dynamics of this area may give the psychrophilic enzymes easier access to the substrate and/or facilitate product release, and, possibly, enable them to accommodate substrates with alternative dimensions and/or orientations (*i.e.*, broader substrate specificity), which are all features commonly associated with cold-adapted enzymes [60,72,75]. Interestingly, while in Rho13 the remaining 43% of the highly flexible residues are found in domain B (**Figure 7a**), I3C6 displays the same percentage of highly flexible residues in domain C (**Figure 7c**). This clearly indicates that, although common trends can be observed between the three psychrophilic amylases studied, each enzyme seems to have developed its own strategy to improve its global/local flexibility and preserve its catalytic activity in cold environments.

The RMSF analysis highlights increased global and local flexibilities on the three cold-adapted amylases *vs.* their mesophilic homologs. These enhanced local flexibilities are mostly concentrated in regions that should play pivotal roles in directing the substrate pathway toward the active site. In addition, other regions with increased flexibility were also found in peripheral areas of the psychrophilic enzymes, which is consistent with previous studies that hypothesized that enhanced flexibility in non-catalytic regions is a common adaptation of psychrophilic enzymes [76]. Interestingly, both Rho13 and Ika2 exhibited some residues with reduced flexibility compared to their mesophilic counterparts (**Figure 7a and 6b**, represented in yellow sticks). This increased rigidity in specific regions likely aids in reducing the entropy barrier for catalysis, highlighting a delicate equilibrium between flexibility and rigidity that may enhance enzyme efficiency in cold climates as discussed in the literature [73,77,78].

To elucidate the largest amplitude collective motions and reduce dimensionality, we performed PCA by diagonalizing the backbone covariance matrices of the concatenated trajectories. Analysis of the first 50 eigenvectors, which together account for over 90% of the total molecular motions, revealed

that the eigenvalues are consistently higher in psychrophilic enzymes than in their mesophilic counterparts. This observation suggests that more significant conformational changes occur in the psychrophilic enzymes. By projecting the trajectory onto the two principal eigenvectors, which represent approximately 50% of the overall motions and delineate a critical subspace for observing the most significant collective motions, we observed that psychrophilic enzymes explore a broader area of conformational space than their mesophilic homologs (**Figure 7d-f**). This increased exploration implies a higher degree of conformational flexibility, likely facilitated by the easier transition over energy barriers between multiple local minima. Such behavior in psychrophilic enzymes could be attributed to the reduced structural stabilizing interactions and compactness, as discussed above.

4. Conclusion

We have isolated and characterized three novel secreted amylases, which despite their common origin in an alkaline and Ca^{2+} -rich environment in Greenland's ikaite columns show significant differences in terms of optimal pH and Ca^{2+} -binding conditions, as well as ability to hydrolyse starch and other carbohydrates. Nevertheless, they share the psychrophilic property of losing activity at temperatures well below those where there is detectable loss in structural integrity. We attribute this to the high levels of conformational flexibility, which facilitate easy binding and accommodation of substrate molecules and exploration of the catalytic energy landscape, but at the expense of structural stability. This is reflected in the reduction of internal polar interactions such as hydrogen bonds and salt bridges, consistent with the general psychrophilic strategy of providing cold activity through conformational flexibility.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. This article contains supporting information with cartoon representations of the three enzymes Rho13, Ika2 and I3C6, PAD elution profiles, size exclusion chromatography data, initial velocity activities, DSF denaturation profiles and MD data on the enzymes' conformational fluctuations and Ca^{2+} binding.

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Abbreviations

The following abbreviations are used in this manuscript:

CD	cyclodextrin
CiD	Circular dichroism
ΔH	Melting enthalpy
dp	Degree of polymerization (glucose units)
DSF	differential scanning fluorometry
G2	maltose
GH	glycoside hydrolase
I3C6	Amylase isolated from metagenomic analysis of ikaite columns
Ika2	Amylase from Bacillaceae isolated from ikaite columns
MBTH	3-methyl-2-benzothiazolinone hydrazone hydrochloride
MD	Molecular dynamics
PCA	Principal component analysis
PDB	Protein Data Bank
R _g	Radius of gyration
Rho13	Amylase from <i>R. psychrophilum</i>
RMSF	root-mean-square-fluctuation
SEC	Size exclusion chromatography
SAXS	Small-angle X-ray scattering
t _m	Melting temperature

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