Physicochemical Characteristics of a Thermostable Gellan Lyase from *Geobacillus stearothermophilus* 98

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A purified thermostable gellan lyase, produced by a thermophilic bacterium, *Geobacillus stearothermophilus* 98, was characterized in relation to its physicochemical properties. The gellan lyase was established to have a molecular weight of 216 kDa, defined by capillary gel electrophoresis. Amino acid analysis revealed high quantities of Lys, His, Ala, Val, Ile, Glx, and Pro residues. The circular dichroism revealed 45% β-structure and practically lack of α-spiral domains. Kinetic studies showed high affinity of the enzyme to gellan as a substrate (K_m = 0.21 μM). The thermal denaturation investigated by cicular dichroism showed a highly cooperative transition with a midpoint (T_m) at about 75 °C. A single product was identified after enzyme action on gellan. Large exothermic aggregation near T_m was observed by differential scanning calorimetry. Two types of gellan lyase crystals were reproducibly isolated.

Key words: Thermostable Gellan Lyase, Amino Acid Composition, Crystals

Introduction

Different chemical compositions and corresponding novel properties of microbial polysaccharides determined the biotechnological interest in their exploitation in recent years. Gellan (linear tetrasaccharide-repeating sequence consisting of D-glucose–D-glucuronic acid–D-glucose–L-rhamnose), synthesized by *Sphingomonas paucimobilis*, is among the microbial exopolysaccharides, finding recently extensive use in food, microbial cultivation media, and pharmaceutical industries (Pollock, 1993; Moritaka *et al.*, 1999; Banik *et al.*, 2000; Rath and Schmidt, 2001). Enzymatically modified gellan with novel physicochemical properties suggests new application fields. Gellan lyase (EC 4.2.2.-) cleaves the bond between D-glucose and D-glucuronic acid by β-elimination type of reaction. Its action lowers the gellan viscosity in solutions and therefore might broaden its current spectrum of application. This enzyme still has no EC number and has not yet been classified in one of the existing 21 polysaccharide lyase families, due to the numerous problems at the level of molecular studies, most of them connected with the large molecular weight of the enzyme.

There are several reports on mesophilic bacterial strains producing gellan lyases (Hashimoto *et al.*, 1996; Sutherland and Kennedy, 1996; Banik and Santhiagu, 2002). The only purified and characterized gellan lyase reported in the literature so far is produced by a Gram-positive mesophilic *Bacillus* sp. GL1 (Hashimoto *et al.*, 1996, 1998; Miyake *et al.*, 2004) and expresses its maximum activity at 45 ºC. As gellan is soluble at temperatures higher than 50 °C, an industrial need for a thermostable gellan lyase is clearly outlined.

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Recently we have reported the biosynthesis of the first thermostable gellan lyase, originally isolated from the obligate thermophilic bacterium *Geobacillus stearothermophilus* 98 with a maximum activity at 70 °C (Derekova *et al.*, 2006). In the present study we report certain physicochemical properties, namely molecular weight, amino acid composition, secondary structures, and thermostability of the gellan lyase from *Geobacillus stearothermophilus* 98.

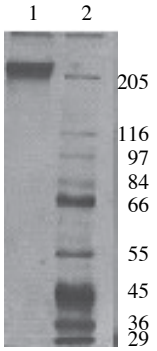

Fig. 1. SDS-PAGE of *G. stearothermophilus* 98 gellan lyase; lane 1, purified gellan lyase (5 μg); lane 2, marker proteins (Sigma), wide molecular weight range.

Results and Discussion

Purification scheme

The purification scheme of the thermostable gellan lyase, applied during the current study, was principally similar to biochemical steps already reported (Derekova *et al.*, 2006) with the modifications reported in Experimental. The applied modifications in the purification scheme of the thermostable gellan lyase were determined by next considerations. Ammonium sulfate precipitation was introduced to enable the treatment of large amounts of culture supernatant provided by continuous fermentation. The next hydrophobic interaction chromatography (HIC) step permitted to desalt the samples avoiding dialysis which otherwise leads to important loss of enzyme activity. The addition of 5% DMSO to active fractions at all stages was an important modification of the previously published purification scheme for the protein as this polar aprotic solvent impeded precipitation of the purified protein and permitted acquisition of higher specific activities of the enzyme.

The total lyase activity in the cultural medium was 0.03 U/ml (specific activity of 0.11 U/mg protein) before the purification process. Gellan lyase from *G. stearothermophilus* 98 was purified ninefold and a final yield of 40.5% was reached. For comparison, a final yield of 5.84% was reached after purification of the precursor gellan lyase protein from *Bacillus* sp. GL1 and of 6.34% for its mature form (Hashimoto *et al.*, 1998).

Molecular weight investigations

The electrophoretically homogeneous enzyme showed a molecular weight of about 220 kDa by SDS-PAGE (Fig. 1). The molecular weight of the purified thermostable gellan lyase defined by capillary gel electrophoresis (CGE) (Fig. 2) was

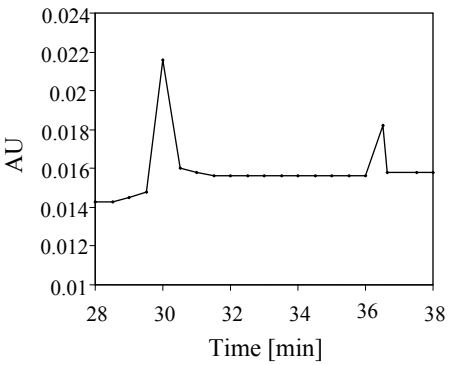

Fig. 2. CGE analysis of the gellan lyase molecule using the Beckman eCAP SDS 14–200 kit/capillary at 214 nm (UV detector). The capillary was calibrated prior to sample loading with a standard mix as described in Experimental. Peak 1 corresponds to the mobility marker Orange G and peak 2 to the purified gellan lyase. The peak of the marker Orange G was eluted at 30.13 min while the peak of the thermostable gellan lyase was eluted at 36.18 min.

similar (216 kDa). A second band with a molecular weight of 120 kDa was observed after preservation of the electrophoretically homogeneous enzyme at –20 °C for a longer period (more than two months). All further investigations were performed with the 220 kDa fraction. Two forms of gellan lyase with molecular weights corresponding to 260 and 130 kDa were observed for the gellan lyase from the mesophilic *Bacillus* sp. GL1 and were explained by enzyme maturation (Hashimoto *et al.*, 1998) The high molecular weight is a common feature of enzymes from *G. stearothermophilus* 98 and *Bacillus* sp. GL1, and a high molecular weight seems to be typical for this group of enzymes; however, further descriptions

of other gellan lyases could confirm this suggestion.

Amino acid composition of gellan lyase from Geobacillus stearothermophilus 98

The results of the amino acid composition analysis of the electrophoretically homogeneous gellan lyase are shown in Table I. The total number of the amino acid residues was 2083 and the estimated molecular weight was 235.2 kDa. Seven amino acid residues were represented in highest quantities: Lys, His, Ala, Val, Ile, Glx, and Pro. These results are in good agreement with the results for other properties of the thermostable gellan lyase. The increased presence of His and Pro residues reflects their significant role for β-structure formation, confirmed by the circular dichroism (CD) spectrum, and the Gly residue content for U-turns in the molecule. Some amino acid residues like Lys, His, Val, Ile, and Pro determined in the enzyme from *G. stearothermophilus* 98 were in higher quantities than the protein from the mesophilic *Bacillus* sp. GL1. These amino acids were generally detected with higher frequency in thermophilic proteins compared to mesophilic ones. In some cases this fact is connected to the predominance of G+C in their codons (Pro), or to the enhanced ability to create hydrogen bonds (His). The presence of a high amount of Lys residues (17.7%) leads to the assumption of a basic character of the *G. stearothermophilus* 98 enzyme molecule in contrast to an increased number of negatively charged residues (Asp + Glu – 10.8%) of the *Bacillus* sp. GL1 enzyme (Hashimoto *et al.*, 1998). The charged amino acids were present in higher numbers of residues in the thermostable gellan lyase molecule in comparison with the mesophilic enzyme. In case of the endo-β-1,4-xylanase from *Geobacillus stearothermophilus* 236 such an increased frequency of charged amino acids was reported to improve a network of intramolecular interactions thus influencing and augmenting the thermostability (Jeong *et al.*, 2007). The significant amount of the hydrophobic amino acids Ala and Val could explain the tendency of the enzyme molecule to aggregate, a problem during enzyme storage partially resolved by the addition of DMSO.

Table I. Established amino acid composition of the gellan lyase from *Geobacillus stearothermophilus* 98 and *Bacillus* sp. GL1 (Prot Param082833_BACSP).

Amino acid	*G. stearothermophilus* 98 gellan lyase		*Bacillus* sp. GL1 gellan lyase	
	Residue number	Content (%)	Residue number	Content (%)
Asx	48	2.3	281	11.4
Glx	36	6.5	225	9.1
Ser	42	2.0	157	6.3
Gly	126	6.0	190	7.7
His	317	15.2	34	1.4
Arg	58	2.8	89	3.6
Thr	30	1.4	174	7.0
Ala	257	12.3	378	15.3
Pro	132	6.3	112	4.5
Cys	24	1.2	0	0
Tyr	16	0.8	91	3.7
Val	211	10.1	174	7.0
Met	16	0.8	46	1.9
Ile	141	6.8	95	3.8
Leu	80	3.8	232	9.4
Phe	46	2.2	64	2.6
Lys	368	17.7	101	4.1
Trp	35	1.7	32	1.3
Total	2083	100	2475	100

Secondary structures

The CD spectrum of the gellan lyase in the far-UV region (195–260 nm) presented in Fig. 3 shows a large negative band with a minimum at 214 nm, indicating a dominant contribution of β-type secondary structure and a small content of α-helix structures.

Based on the CD spectrum of the gellan lyase, three different theoretical methods have been utilized to estimate the content of different secondary structures in the enzyme. The results of the calculations are presented in Table II. The analysis of these results with two of the programs (CONTIN and CDSSTR) indicated similar values of secondary structure content in the gellan lyase: about 40% for β-type structure and about 54% for turns. Similarly, coils and β-stranded sections prevail (73% and 17% correspondingly) in the structural model of the thermostable gellan lyase sequence, built by the Phyre program (Bennett-Lovsey *et al.*, 2008) while a small amount of α-helixes (10%) is predicted (data not shown).

Kinetics and stability

The hydrolysis of gellan followed Michaelis-Menten kinetics. The K_m value was found to be

Table II. Circular dichroism methods for analysing the gellan lyase conformation.

Program	CONTIN (Ref. prot. set: SMP56)	CDSSTR (Ref. prot. set: SMP56)	SELCON3 (Ref. prot. set: SMP56)
Right helix (non-distorted)	.016	.046	.269
Distorted helix	.006	.052	.279
Regular strand	–.033	.030	.556
Distorted strand	.129	.118	.161
Turn	.221	.226	.197
Disordered	.318	.313	.085

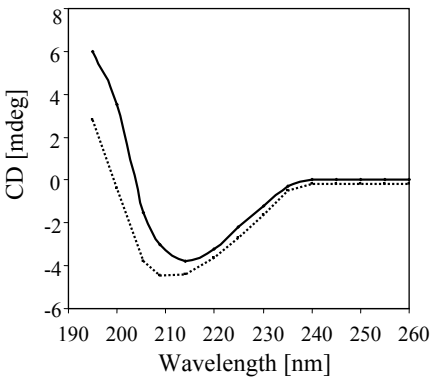

Fig. 3. CD spectra of native enzyme (—) and gellan lyase after reversible denaturation (**---**). The spectra were done in 20 mM phosphate buffer, pH 7.2, at 20 °C. The enzyme concentration was 0.4 μM.

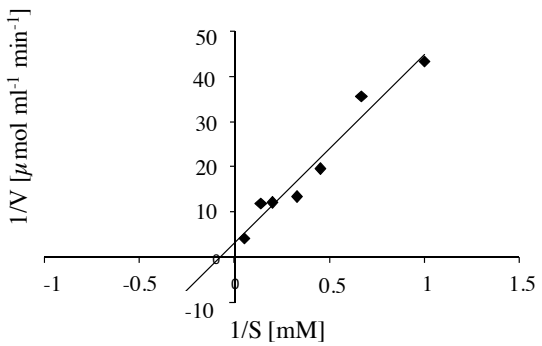

Fig. 4. Lineweaver-Burk plot. The results presented were obtained by reducing sugar analysis. *V*, reaction velocity; *S*, substrate (gellan) concentration.

0.21 μM gellan, and maximum hydrolysis occurred at the V_{max} value of 3.11 μmol min^{-1} (Fig. 4).

The thermal denaturation of the gellan lyase was investigated by means of CD studies. The thermal denaturation curve of the enzyme at 214 nm showed a highly cooperative transition with a midpoint (T_m) at about 75 °C. This value is in good agreement with already reported thermostability of the enzyme – the half life is 1 h at 70 °C, and its temperature optimum is 70 °C (Derekova *et al.*, 2006). As the process of denaturation was practically reversible, we fitted the data to a two-state denaturation process. The CD signal in the near-UV range also showed a transition step within the same temperature range, indicating that the tertiary and secondary structure unfolding were parallel.

The thermal stability of the gellan lyase was further characterized using differential scanning calorimetry (DSC). At pH 7.2 and a protein concentration above 1 mg ml^{-1} large exothermic aggregation occurred near T_m (75 °C) rendering that the unfolding transition is irreversible. At concentrations below 1 mg ml^{-1} the DSC endotherms were complete and reversible, but the signal was very weak. Provided calorimetric and CD spectroscopic data demonstrate valuable thermokinetic evidence for the stability of the enzyme.

Products of gellan lyase action

Gellan lyase cleaves the gellan molecule to tetrasaccharide-repeating units of glucuronic acid–glucose–rhamnose–glucose through the reaction of β-elimination. The lyase activity of the enzyme from *G. stearothermophilus* 98 was confirmed spectrophotometrically by continuously increasing the absorbance at 235 nm (Derekova *et al.*, 2006). The formation of only one product after the action of electrophoretically homogeneous gellan lyase was observed on a TLC plate (Fig. 5, lane 3). Its mobility was different from the mobility of the gellan components, such as D-glucose, L-rhamnose, and D-glucuronic acid (Fig. 5, lane 2), and placed on the position identical to that of the product from *Bacillus* sp. GL1 gellan lyase action (Hashimoto *et al.*, 1998).

Two spots were visualized as products derived from gellan by action of the unpurified enzymes in the supernatant (15 h) (Fig. 5, lane 1). Except the observed tetrasaccharide, low molecular weight

products migrating in a similar way like glucose and glucuronic acid were well visible. These results suggested that gellan was depolymerized first by gellan lyase and the received tetrasaccharide further hydrolyzed by certain exoenzymes which were found outside the cell in the stationary phase. Gellan-induced synthesis of all three enzymes participating in gellan degradation (gellan lyase, β-D-glucosidase and α-L-rhamnosidase) was already observed (Hashimoto *et al.*, 1996).

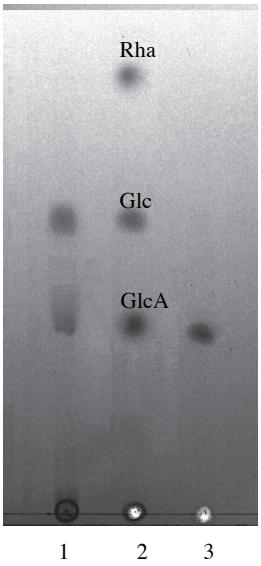

Fig. 5. TLC analysis of the products obtained by degradation of gellan by *G. stearothermophilus* 98 gellan lyase; lane 1, degradation of gellan by the extracellular enzymes fraction presented in the supernatant of *G. stearothermophilus* 98 in the stationary phase of growth (30 mU/ml, 20 μl); lane 2, standard [D-glucuronic acid (GlcA), D-glucose (Glc), L-rhamnose (Rha)] (10 μl); lane 3, degradation of gellan by purified gellan lyase (0.81 U/ml, 20 μl).

Crystallization

Small crystals grew under the following four conditions of the screens: (a) 0.2 M Zn acetate, 0.1 M sodium cacodylate, pH 6.5, and 18% PEG 8000; (b) 0.01 M $ZnSO_4$, 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 6.5, and 25% PEG 550 in MME (100 mM MES, 1 mM $MgCl_2$, 1 mM EGTA [(ethylene glycol his) (~-aminoethylether)-*N,N,N'N'*-tetraacetic acid)], pH 6.75); (c) 0.2 M $CaCl_2$, 0.1 M sodium acetate, pH 4.6, and 20% isopropanol; (d) 0.2 M magnesium formate. The quality of the crystals was optimized by changes of the concentration of the precipitants as well as the drop volumes.

Two types of crystals of gellan lyase were finally observed: first, at 0.2 M $CaCl_2$, 0.1 M sodium acetate, pH 4.6, and 20% (v/v) isopropanol (Fig. 6A). Two of the dimensions of this type of plate-like single crystals are: 60–80 μm *vs.* 150–200 μm. Second, at 0.01 M $ZnSO_4$, 0.1 M MES, pH 6.5, and 15% (w/v) PEG 550 in MME (Fig. 6B) we observed bundle-like crystal rods.

These two types of crystals are well reproducible and could be successfully utilized for further X-ray structure determinations. The crystal characterization of the polysaccharide lyases is still in an opening stage. The structures of few polysaccharide lyases acting endolytically have recently been determined (Mayans *et al.*, 1997; Huang *et al.*, 1999; Yoon *et al.*, 1999; Yamasaki *et al.*, 2005), in this context a crystallographic analysis has been performed for xanthan lyase (Hashimoto *et al.*, 2003; Maruyama *et al.*, 2005). Until now the crystals from gellan lyases have not been reported.

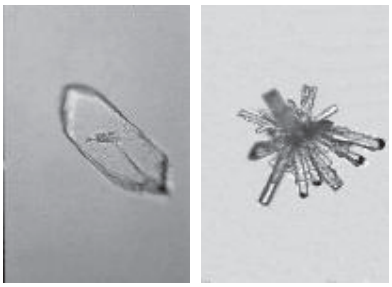

Fig. 6. Gellan lyase crystals. (A) Plate-like single crystal of gellan lyase. The crystal was observed in 0.2 M $CaCl_2$, 0.1 M sodium acetate, pH 4.6, and 20% (v/v) isopropanol. (B) Bundle-like crystal rods of gellan lyase. The crystals were observed in a solution containing 0.01 M $ZnSO_4$, 0.1 M MES, pH 6.5, and 15% (w/v) PEG 550 in MME.

Experimental

Bacterial strain

The strain *Geobacillus stearothermophilus* 98 was isolated from a Bulgarian hot spring at a sampling temperature of 72 °C as previously described (Derekova *et al.*, 2006). The strain was cultivated and preserved in peptone/yeast extract medium (0.2% and 0.1% correspondingly).

Enzyme assays

The gellan lyase activity was assayed by both, reducing sugar analysis according to the dinitrosalicylic acid method (Miller, 1959) and spectrophotometric detection at 235 nm of unsaturated bonds accumulation (Derekova *et al.*, 2006). Lowry's method was used for quantitative determination of the protein content in samples (Lowry *et al.*, 1951).

Purification scheme

The enzyme was purified as previously described (Derekova *et al.*, 2006) applying some modifications. The culture medium and all active fractions were stored at 4 °C with addition of 5% DMSO as a stabilizing agent. The supernatant was salted out overnight with ammonium sulfate to 40% and then to 60% of saturation and centrifuged for 1 h at 4 °C, 15000 × *g*. The obtained pellet was resuspended in 6 mM Tris buffer (pH 8.0) + 1 M KCl, and HIC was performed upon a phenyl sepharose column. An elution gradient was applied from 1 M to 0.5 M KCl in 6 mM Tris buffer, pH 8.0. Next steps followed the original procedure (Derekova *et al.*, 2006). The final purity of the enzyme preparation was checked by SDS-PAGE analysis (7% resolving gel). The silver staining procedure applied was according to the method of Heukeshoven and Dernick (1985).

Capillary electrophoretic analysis

The molecular weight of the purified gellan lyase molecule was studied by CGE on a Beckman Coulter P/ACE MDQ system (Fullerton, CA, USA), with an SDS-coated capillary, protein standards, and specific buffers from the Beckman eCAP SDS 14–200 kit, according to the manufacturer's instructions. The capillary was calibrated prior to sample analysis with a standard mix of proteins, 14.2–205 kDa. The equation of the standard line obtained for the mix of proteins and used for calculation of the gellan lyase molecular weight had the correlation coefficient $R^2 = 0.9654$. A commercial solution of Orange G (Beckman Coulter) was added to both sample and calibration protein mix as an electrophoretic mobility marker.

Determination of amino acid composition

The sample of the purified enzyme was hydrolyzed using 6 M HCl during 24 h at 106 °C, without taking special precautions to prevent (partial) hydrolysis of Ser, Thr, Cys, and Trp. The amino acid composition analysis was carried out on a 420 Derivatizer linked to an 130 A Separation System (Applied Biosystems, Foster City, CA, USA), using the precolumn derivatization method with phenyl isothiocyanate, with on-line separation of the PTC × amino acid derivatives on a Brownlee PTC-C18 reversed phase column (2.1 × 250 mm; Applied Biosystems). The analysis was carried out at the Laboratory for Protein Biochemistry and Biomolecular Engineering, University of Gent, Belgium. The availability of tryptophan residues was evaluated from the UV absorption spectra using the coefficients $\varepsilon(280\text{ nm}) = 1490$ and $5500\ \text{M}^{-1}\ \text{cm}^{-1}$ for Tyr and Trp residues, respectively (Pace *et al.*, 1995).

Kinetic study

The effect of the substrate concentration (S, Gelrite) on the reaction rate was assayed using a standard enzyme assay. The Michaelis-Menten constant (K_m) and the maximum velocity for the reaction (V_{max}) were determined by linear regression analysis of Lineweaver-Burk plots from at least five substrate dilutions.

Thin-layer chromatography

Products of gellan degradation by purified gellan lyase and other gellan-degrading enzymes present in the supernatant were detected by thin-layer chromatography. The reactions were performed at 70 °C for 60 min in 0.1 ml of a mixture containing 0.05 ml 0.5% gellan in 20 mM phosphate buffer, pH 7.0, and 0.05 ml solution of purified gellan lyase or culture supernatant of *G. stearothermophilus* 98 in the stationary phase (15 h) of growth as a source of extracellular enzymes fraction. Aliquots (20 μl) of the reaction mixtures were spotted on Silica gel 60/

Kieselgel aluminium plates (Merck). The plates were developed in the solvent system *n*-propanol/ethylacetate/water [7:1:2 (v/v/v)]. A mixture of authentic D-glucuronic acid (0.5%), D-glucose (0.5%), and L-rhamnose (0.5%) [1:1:1 (v/v/v)] was used as a standard. The plates were air-dried in a hood and then heated at 110 °C for 10 min for visualization of the spots.

Spectroscopic methods

Absorption spectra of gellan lyase were recorded on a Shimadzu UV-3000 UV-VIS spectrophotometer, and a Perkin Elmer LS-5 spectrofluorimeter was used for the fluorescence spectra. The emission spectra were corrected using a standard tungsten lamp, whereas the excitation spectra were corrected with rhodamine B.

The CD spectra of gellan lyase (from 195 to 260 nm) were acquired on a Jasco 715 spectropolarimeter equipped with a device for automatic temperature control. Estimation of the gellan lyase secondary structure from CD spectra has been done by comparison of the results from three different methods: CONTIN, CDSSTR, and SELCON3 (Greenfield, 1996).

CD measurements monitoring the ellipticity at 214 nm as a function of temperature (1 °C min^{-1}) determined the denaturation curves for 0.4 μM protein solution in 20 mM phosphate buffer, pH 7.2. Supposing that thermal unfolding is an equilibrium process, one can fit the experimental data to a two-state model and estimate the thermodynamic parameters of unfolding.

Microcalorimetric study

DSC endotherms were obtained using the Micro-Cal DSC equipment. A gellan lyase solution (1 mg ml^{-1}) was dialyzed overnight at 4 °C against 20 mM phosphate buffer, at pH 7.2. Samples were degassed under vacuum for 10 min with gentle stirring prior to being loaded into the cell of the calorimeter (0.5 ml). Samples held *in situ* under a constant external pressure to avoid bubble formation and evaporation at high temperatures were equilibrated 30 min at 25 °C, then heated at a constant scan rate of 1 °C min^{-1}. Experimental data was collected with a 16-s filter, and the instrument baseline was used for noise substraction in scans of the same buffer, prior to data analysis.

Crystallization study

Crystallization tests were performed with two different commercial screens (the PEG Suite and the Classics Suite; Nextal Biotechnology, Montreal, Quebec, Canada), each comprising 96 conditions. The protein concentration was 5.4 mg ml^{-1}. The use of a higher concentration was limited by the precipitation process in the protein solution. Initial crystals were obtained from the stock solution by the hanging-drop vapour-diffusion method at 18 °C by mixing 1 μl protein solution with 1 μl of a 500-μl reservoir solution. Controls without protein were run simultaneously.

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