Cloning, production, purification and preliminary crystallographic analysis of a glycosidase from the food lactic acid bacterium *Lactobacillus plantarum* CECT 748^T

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Abbreviations: Bgl, His_6-tagged β-galactosidase from *L. plantarum*; glucosidase; CTAB, cetyltrimethylammonium bromide; galactosidase; crystallization;

ABSTRACT

In recent years, the exquisite stereoselectivity and high efficiency of carbohydrate-processing enzymes have been exploited for many biotechnological applications, including flavour enhancement in foods. With the aim to produce and structurally characterize a glycosidase from the food lactic acid bacterium *Lactobacillus plantarum*, we have cloned and heterologously expressed the *bgl* gene (lp_3629) in *E. coli.* The recombinant protein containing an amino terminal His_6 tag (Bgl) has been produced in a soluble form. Purified recombinant enzyme shows galactosidase activity against 4-nitrophenyl β-D-galactopyranoside but not glucosidase activity. Analytical size-exclusion gel filtration chromatography reveals that Bgl behaves in solution as a mixture of monomeric and a high-molecular weight assembly. Purified Bgl has been crystallized by the hanging-drop vapour-diffusion method at 18 ºC. Diffraction data have been collected at ESRF to a resolution of 2.4 Å. The crystals belong to the space group *C*2 with unit-cell parameters a = 196.7, b = 191.7, c = 105.9, β = 102.7º. The structure refinement is in progress.

Introduction

In the past decades, the need for new carbohydrate materials and the development of glycomics have provided a boost to carbohydrate chemistry. Oligosaccharides can be prepared by classical organic chemistry using expensive and tedious methods. However, as an alternative, enzymatic synthesis, ideally in a stereo- and regio-specific manner, is a valued option. Elegant glycosidic bond formation can be accomplished by using glycosidases. In recent years, the recent advances in carbohydrate synthesis by glycosidases are based on two complementary approaches: the use of wild-type enzymes with engineered substrates, and mutant glycosidases [1].

The extensive variety of stereochemistry of carbohydrate is paralleled by a large multiplicity of the enzymes involved in their metabolism. The IUB Enzyme Nomenclature is based on the type of reaction that enzymes catalyse and on their substrate-specificity. For glycosidases or glycosyl hydrolases (EC 3.2.1.-), the first three digits indicate enzymes hydrolysing O-glycosyl linkages whereas the last number indicates the substrate and sometimes reflects the molecular mechanism. However, at least in the case of glycosyl hydrolases, such a classification does not necessarily reflect (and was not intended to) the structural features of the enzymes. In fact, a classification based primarily on the substrate cannot take into account evolutionary events such as divergence (which can result in specificity, and sometimes reaction-type, changes) or convergent evolution, which may force polypeptides with different folds to catalyse the same reaction on the same substrate. Another problem with the EC classification is that it is not appropriate for enzymes showing broad specificity (i.e. that act on several substrates) [2].

Among the glycosidases, β-glucosidases have been subject of much work because of their importance in numerous biological processes and in biotechnological applications [3], such as food detoxification [4], biomass conversion [5] and over the past decade, flavour enhancement in beverages [6]. Indeed the intensive research carried out over the past two decades has demonstrated that, in a great number of fruit and other plant tissues, important flavour compounds accumulate as non-volatile and flavourless glycoconjugates, which make up a reserve of aroma to be exploited

[7,8]. Therefore, hydrolysis of these glycosides leads to the liberation of volatiles. Wine aroma and flavour are influenced by grape-derived compounds and by the microorganisms which are present during winemaking. Recent reports have shown that lactic acid bacteria strains involved in wine malolactic fermentation possess β-glucosidase activity [9,10]. Likewise, it has been described that *Lactobacillus plantarum*, a wine-related lactic acid bacteria species, possess a putative β-glucosidase (Bgl) which expression is regulated by abiotic stresses such temperature, ethanol, and pH [11].

As glycosidases, and specifically β-glucosidases, are key enzymes in the enzymatic release of aromatic compounds from glycoside precursors present in fruits and fermenting products, and the application of these enzymes in a well-defined manner requires a large-scale production of the enzymes and a detailed knowledge of their structure, we decided to produce and physically characterized the putative β-glucosidase (Bgl) previously described in the food *L. plantarum* species.

Materials and methods

Gene cloning and Bgl protein production

The *bgl* gene coding for a putative β-glucosidase (lp_3629) from *Lactobacillus plantarum* CECT 748^{T} (ATCC 14917^{T}) was PCR-amplified by Hot-start Turbo *Pfu* DNA polymerase by using the primers 371 (5′- *CATCATGGTGACGATGACGATAAGat*ggtagagtttccggaaggctttg) and 372 (5′- *AAGCTTAGTTAGCTATTATGCGTA*tcaaaacccattccgttccccaagc) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *bgl* gene sequence are written in lowercase letters). The 1.4-kb purified PCR product was inserted into the pURI3 vector by using the restriction enzyme- and ligation-free cloning strategy described previously [12,13]. Expression vector pURI3 was constructed based on the commercial expression vector pT7-7 (USB) but containing the following leader sequence MGGSHHHHHHGDDDDKM consisting of an N-terminal methionine followed by three spacer amino acids, a six-histidine affinity tag, a spacer glycine residue, and the five-amino acid enterokinase recognition site. Thus, the final recombinant Bgl would possess 477 amino acid residues with a molecular weight of 54.5 kDa. *E. coli* DH5α cells were transformed, recombinant plasmids were isolated and those containing the correct insert were identified by restriction-enzyme analysis, verified by DNA sequencing and then transformed into *E. coli* JM109 (DE3) cells for expression.

Cells carrying the recombinant plasmid, pURI3-Bgl, were grown at 37 ºC in Luria-Bertani media containing ampicillin (100 μg ml^{-1}), until they reach an optical density at 600 nm of 0.4 and induced by adding IPTG (0.4 m*M* final concentration). After induction, the cells were grown at 22 ºC during 20 h and collected by centrifugation.

Protein Purification

The bacterial cell pellet was resuspended in Tris buffer (Tris-HCl 20 mM, pH 8.0 containing NaCl 100 mM) and homogenized by French Press. The lysate was centrifuged in an SS34 rotor at 20000 rpm using a Sorvall centrifuge for 30 min. at 4 ºC to remove the cell debris. The supernatant was then applied to a HisTrap FF Ni-affinity column (GE Healthcare). Non-specific adsorbed materials were washed off with Tris buffer containing 10 mM imidazole. Bgl was purified in a linear gradient of imidazole (10-500 mM) prepared in Tris buffer with an AKTA-prime (Pharmacia). Major peak fractions containing Bgl were pooled and dialyzed against 10 mM NaCl in 20 mM Tris-HCl buffer, pH 8.0. The protein was further purified by ion exchange chromatography on a HiTrap Q HP column (GE Healthcare) in a linear gradient of 10-500 mM NaCl in 20 mM Tris-HCl buffer, pH 8.0. Fractions containing Bgl were pooled and dialyzed against 100 mM NaCl, 2 mM DTT in 20 mM Tris-HCl buffer, pH 8.0. The sample was then concentrated and applied on a Superdex 200 prep grade column (GE Healthcare). The purified recombinant material thus obtained was concentrated with YM-10 Centricon filters (Millipore) to 9 mg/ml in 20 mM Tris-HCl pH 8.0, containing 100 mM NaCl, 2 mM DTT, and 0.04 % (w/v) sodium azide. Protein concentration was determined by UV-VIS absorbance measurements with a Nanodrop® ND-1000 spectrophotometer, using an extinction coefficient of 2.15 (1 mg ml^{-1}, 1 cm, 280 nm).

Enzyme assay

Glycosidase activity was measured by determining the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-galactopyranoside at 37 ºC after 10 min incubation. The reaction mixture (500 µl) consisted of 20 mM Tris-HCl pH 8.0, containing 100 mM NaCl and 2 mM DTT. The reaction was stopped by adding and equal volume of 0.5 M glycine/NaOH buffer pH 9.0, containing 2 mM EDTA. The colour formation was measured at 420 nm. Enzyme activity was measured as a function of the liberated *p*-nitrophenol, determined by the absorbance at 420 nm. One

unit of β-galactosidase activity was defined as the amount of enzyme liberating 1µmol of *p*-nitrophenol per minute under the above specified conditions.

Analytical size exclusion chromatography

Analytical size exclusion chromatography was performed on a Superdex 200 10/300 GL Tricorn column (GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 2 mM DTT and 0.04 % (w/v) sodium azide. The column was calibrated with thyroglobulin (667 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol deshydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), sperm whale myoglobin (17 kDa) and vitamin B12 (1.3 kDa) in the same buffer. The size of Bgl was determined from its K_{av} value ($K_{av} = (V_e - V_0)/(V_t - V_0)$; V_e: elution volume; V_0: void volume; V_t: total volume of the column) by interpolation in a calibration semilog plot of the molecular mass of the standard proteins *versus* their K_{av} values.

Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on a Finnigan LCQ Deca ion trap Mass Spectrometer (Thermo Electron, San José, CA, USA) was used to determine the molecular mass of Bgl. Mass spectra were recorded in full scan mode (*m/z* = 450-2000) and protein peaks detected were deconvoluted using the BIOMASS deconvolution tool from BioWorks 3.1 software (Thermo Fisher Scientific).

Crystallization and preliminary X-ray diffraction studies

Initial crystallization conditions of Bgl at 291 K were determined using the sparse matrix method [14] with commercial screens from Hampton Research (Riverside, CA) and Qiagen in crystallization trials by the sitting-drop vapour-diffusion method in Innovaplate SD-2 96-well plates. Crystals of Bgl appeared in several conditions containing PEG 3350. Optimization of the crystallization conditions with additive and detergents screens from Hampton Research (Riverside, CA) rendered high quality diffraction crystals in hanging drops containing 1 μl of the protein solution (9 mg/ml in 20 mM Tris-HCl, pH 8.0 with 0.1 M NaCl, 2 mM DTT and 0.04% (w/v) sodium azide), 1 μl of reservoir solution (15% (w/v) PEG 3350, 0.2 M di-ammonium phosphate, 0.1 M sodium cacodylate, pH 6.4, 2 mM DTT) and 1 μl of detergent CTAB from Detergent Screen 1 from Hampton Research (Riverside, CA). Crystals for X-ray analysis were transferred to an optimized cryoprotectant solution (reservoir solution plus 25% (v/v) 2-methyl-2,4-pentanediol) for ~5 secs and then cryocooled at 100 K in the cold nitrogen-gas stream. Diffraction data were recorded on an ADSC Q210r CCD detector (Area Detector Systems Corp.) at beamline BM16 at the European Synchrotron Radiation Facility (ESRF) (Grenoble, France). The images were processed and scaled using MOSFLM [15] and SCALA from the CCP4 program suite [16]. Intensities were converted to structure-factor amplitudes using TRUNCATE also from the CCP4 suite [16]. Data-collection statistics are given in Table I.

Structure determination

The crystal structure of Bgl was determined by the molecular replacement method with PHASER [17]. The atomic coordinates of the engineered β-glucosidase from soil metagenome (PDB code 3cmj) were used as the search model.

Results and discussion

Gene cloning and Bgl production

Lactic acid bacteria constitute a family of Gram-positive bacteria that are extensively used in the fermentation of raw agricultural products and in the manufacture of a broad variety of food products. Food aroma and flavour are influenced by compounds which could exist as odourless sugar-bound precursors. In *L. plantarum*, a wine lactic acid bacteria species, it has been described the presence of several glycosidase activities [9]. Accordingly, its complete genome sequence revealed the existence of an open reading frame (lp_3629) encoding a putative β-glucosidase, Bgl, that shows high similarity to proteins found in several lactic acid bacteria (LAB) such as *Lactobacillus paraplantarum*, *Lactobacillus pentosus, Pediococcus damnosus* and *Oenococcus oeni* [11]. As this protein could have biotechnological interest, we decided to produce *L. plantarum* β-glucosidase. The 1.4 kb *bgl* gene was PCR amplified from genomic DNA and inserted into the pURI3 vector using the restriction enzyme- and ligation-free cloning strategy described previously [12,13]. The expression vector pURI3-Bgl thus prepared would encode for Bgl with an amino terminal His_6 affinity tag, potentially removable by enterokinase treatment, which has permitted us to obtain crystallization grade purity proteins [18,19].

The deduced amino acid sequence of the final recombinant His_6-tagged Bgl protein has 477 amino acid residues with an estimated molecular weight of 54502 Da. A BLAST comparison against the non-redundant PDB revealed that Bgl shares similarity (sequence identity ~30 %) to glycosidases from the glycosyl hydrolase family 1 (GH1) (**Fig. 1**) whose basic structure is based on a $(\beta/\alpha)_8$ barrel scaffold. From this multiple alignment it can be deduced that neither the number nor the distribution pattern of Cys residues are conserved in these proteins what suggests that they are not directly involved in glycosidase function. Nevertheless, they may be relevant in protein stability, as is the case for Bgl, where the redox state of the Cys residues critically affects Bgl solubility as the absence of the reducing agent DTT in the final protein buffer (~2-3 mM) rendered insoluble material.

Cells extracts were used to detect the presence of hyperproduced proteins by SDS-PAGE analysis. Control cells containing the pURI3 vector plasmid alone did not show expression over the time course analyzed, whereas expression of an additional 55 kDa protein was apparent in cells harbouring pURI3-Bgl. Induction with 0.4 mM IPTG at an OD_{600} of 0.4 was as efficient as with 1 mM IPTG and therefore the lower concentration was selected to increase the production yield of soluble material. Expression levels were highest at ~20 h after induction and were fairly independent of the different temperature values considered (22, 30 and 37º C). The final production yield of soluble Bgl was estimated to be approximately 15 mg/1000 ml liquid culture. SDS-PAGE analysis of Bgl samples at different steps of the purification process are shown in **Fig. 2.**

Glycosidase activity

Despite the extensive description of glycosidase activities in lactic acid bacteria, the corresponding enzymes have been scarcely characterized. The complete genome sequence of *L. plantarum* revealed the presence of an open reading frame, lp_3629, coding for a putative β-glucosidase belonging to the glycosyl hydrolase family 1. GH1 enzymes have a wide range of specificities, including β-glucosidase (EC 3.2.1.21), β-galactosidase (EC 3.2.1.23), 6-phospho-β-galactosidase (EC 3.2.1.85), 6-phospho-β-glucosidase (EC 3.2.1.86), myrosinase or sinigrinase (EC 3.2.1.147), and lactase-phlorizin hydrolase (EC 3.2.1.62/108) activities [2]. In order to biochemically demonstrate that Bgl encodes a functional β-glucosidase we tested 4-nitrophenyl β-D-glucopyranoside as substrate for Bgl. Unexpectedly, this compound was not hydrolyzed by pure Bgl protein. As family 1 glycosidase also include enzymes showing β-galactosidase activity, we incubated pure Bgl in the presence of its corresponding nitrophenyl derivatives, 4-nitrophenyl β-D-galactopyranoside, as potential substrate. The purified enzyme was able to liberate β-galactose and *p*-nitrophenol, with the concomitant appearance of a yellow colour. A specific activity of 670 U/µg was quantified for 4-nitrophenyl β-D-galactopyranoside. Thus, we could prove experimentally that

the lp_3629 encodes a functional β-galactosidase, and not a β-glucosidase. The existence of a number of glycosidases polyspecific families, family 1 among them, indicates that the acquisition of new specificities by glycosyl hydrolases is a common evolutionary event. The divergence of glycosyl hydrolases to acquire new specificities is not unexpected given the stereochemical resemblance between some of their substrates [2]. Moreover, this unexpected result strongly indicated that assignments of function based on genomic data must be verified by experimental data generated by assays of the isolated enzyme; as even when the type of reaction catalyzed by a specific gene product could be predict, it is often unable to establish the substrates used by the enzyme [20].

Oligomeric state of Bgl in solution

The oligomeric state of Bgl in solution has been studied by analytical gel-filtration chromatography on a Superdex 200 10/300 GL Tricorn column in conjunction with SDS-PAGE. The obtained results (**Fig. 3**) indicated that Bgl exists in solution as a mixture of two resolvable species of 390.6 ± 4.3 kDa and 70.0 ± 3.4 kDa (n=3) respectively, with subunits of ~55 kDa. These results compare well with the theoretical masses expected for heptameric (381 kDa) and monomeric (54.5 kDa) Bgl, respectively. Additionally, we have confirmed the molecular weight of monomeric Bgl by MALDI-TOF-MS analysis. The mass value of *m/z* 54,375.0 was observed which is in agreement with the calculated average mass of 54,370.9 for the recombinant Bgl without the amino terminal Met residue. Taken together, these results indicated that Bgl behaves in solution as an associative system between a monomeric species and a well-defined high molecular weight assembly, which is practically displaced to the oligomeric state.

Crystallization and crystal packing

Initial crystals of Bgl obtained in crystallization trials by the sitting-drop vapour-diffusion method grew as hexagonal prisms and small plates coexisting within the same crystallization drops. Crystals appeared in several crystallization conditions containing PEG 3350. After optimization of crystallization conditions to 15% (w/v) PEG 3350, 0.2 M di-ammonium phosphate, 0.1 M sodium cacodylate, pH 6.4, 2 mM DTT with detergent CTAB, large and high-quality diffraction plates (0.1x 0.3 x 0.5 mm^3) were obtained (**Fig. 4A**). The collected data set is 94.4 % complete up to 2.35 Å resolution (**Fig. 4B**) and merged with an overall R_{sym} of 12.8%. The crystals belong to the monoclinic space group C2 with unit cell dimensions a = 196.7 Å, b = 191.7 Å, c = 105.9 Å, α = 90, β = 102.74, γ = 90.

The structure of Bgl from *L. plantarum* has been determined using the molecular replacement method with PHASER from the CCP4 suite of programs, with the atomic coordinates of engineered β-glucosidase from soil metagenome (PDB code 3cmj) as the search model. Other models (see **Fig. 1**) did not permit finding a molecular replacement solution. The asymmetric unit of the monoclinic crystal is made up of six Bgl polypeptide chains with a Matthews´ coefficient of 2.9 $Å^3$/Da (solvent content 57.5%). Analysis of the crystal protein packing indicates that Bgl may form hexamers made up of two stacked trimers (**Fig.5**). This result strongly suggests that the high-molecular weight assembly identified with analytical gel-filtration experiments corresponds to a hexameric Bgl. The structure refinement of Bgl is in progress.

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Legends to the figures

Fig.1. Multiple amino acid sequence alignment of Bgl with glycosidases with known three dimensional structure which show similarity to Bgl as revealed by BLAST. Highly conserved positions are shown in *blue*, and Cys residues are in *green*. BGAL: *L. plantarum* Bgl; 3CMJ: β-glucosidase from a soil metagenome; 1Od0: β-glucosidase from *Thermotoga maritima*; 2RGL: β-glucosidase from rice; 1BGA: β-glucosidase from *Bacillus polymyxa*; 1PBG: 6-phospho-beta-galactosidase from *Lactococcus lactis*; 2DGA: β-glucosidase from wheat; 1HXJ: β-glucosidase from maize; 1GNX: β-glucosidase from *Streptomyces sp.*; 1QOX: β-glucosidase from *Bacillus circulans sp. alkalophilus.*

Fig.2. SDS-PAGE analysis of Bgl samples at different steps of the purification process. Lane 1, broad range (Bio Rad) molecular weight markers; lane 2, soluble fraction from *E. coli* JM109(DE3) cells carrying the plasmid pURI3-Bgl after cell disruption; lane 3, pooled fractions containing Bgl eluted from the His-Trap FF Ni-affinity column; lane 4, pooled fractions containing Bgl eluted from the HiTrap Q HP column; lane 5, pooled fractions containing Bgl eluted from Superdex 200.

Fig.3. Analytical gel-filtration studies of Bgl. Two peaks of hexamer and monomer were observed. The elution profile of Bgl is shown together with the elution positions of some standard proteins. The scale at the *bottom* indicates the elution time. *Inset*, semilog plot of the molecular mass of all the standard proteins used *versus* their K_{av} values.

Fig.4. (A) Optimized crystals of recombinant Bgl from *Lactobacillus plantarum* grown at 291 K in 15% (w/v) PEG 3350, 0.2 M di-ammonium phosphate, 0.1 M sodium cacodylate, pH 6.4, 2 mM DTT with detergent CTAB. (B) X-ray diffraction image of Bgl collected on beamline BM16 at

ESRF (Grenoble, France). The crystal-to-detector distance was 213 mm, and wavelength was 0.9794 Å. Diffraction maxima extended beyond 2.3 Å resolution. The edge of the detector corresponds to 2.2 Å resolution.

Fig.5. Hexameric assembly of Bgl within the monoclinic crystals. Analysis of the protein packing reveals a hexameric assembly resulting from the stacking of two trimers of Bgl, namely, the hexamer is a dimer of trimers. The six molecules of Bgl are shown as ribbon models. Each trimer is represented by a different color. The figure has been prepared with PyMol [21].

Tables

Table 1
Data-collection and processing statistics.

Beamline	BM16 (ESRF)
Wavelength (Å)	0.9794
Space group	*C*2
Unit-cell parameters (Å)	a= 196.7, b = 191.7, c = 105.9 α = 90º, β = 102.7º, γ = 90º
V_M (Å^3 Da^{-1})	2.9
Monomers per ASU	6
Solvent content (%)	57.5
Resolution range (Å)	67.73-2.35[2.48-2.35]
R_{sym}(%)	12.8[59.8]
Mean I/σ(I)	8.8[1.6]
No. of measured reflections	441599
No. of unique reflections	149622 [22353]
Completeness (%)	94.4 [94.4]
Multiplicity	3.0 [2.9]

Values for the highest resolution shell are given in square brackets.

Figure

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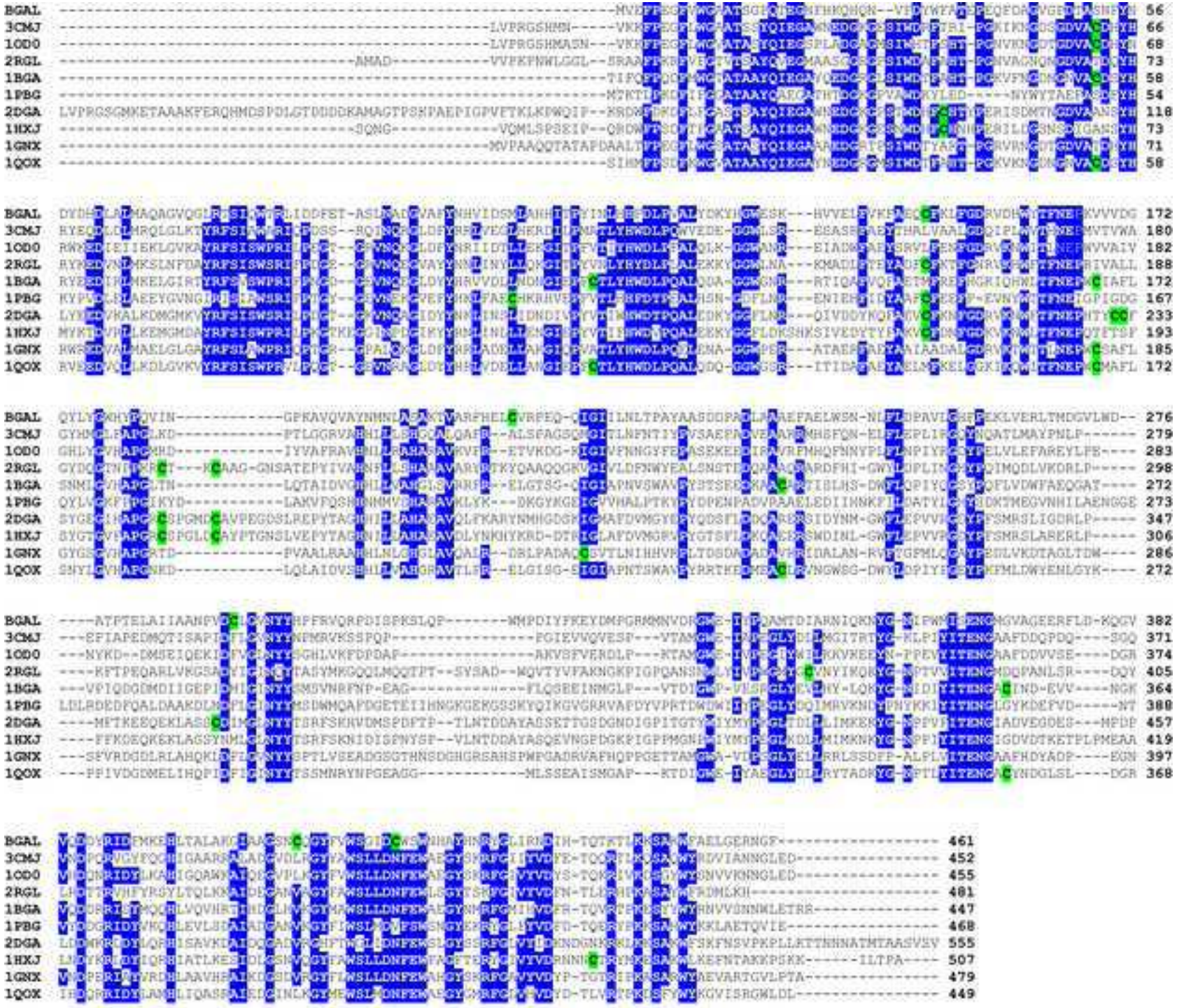

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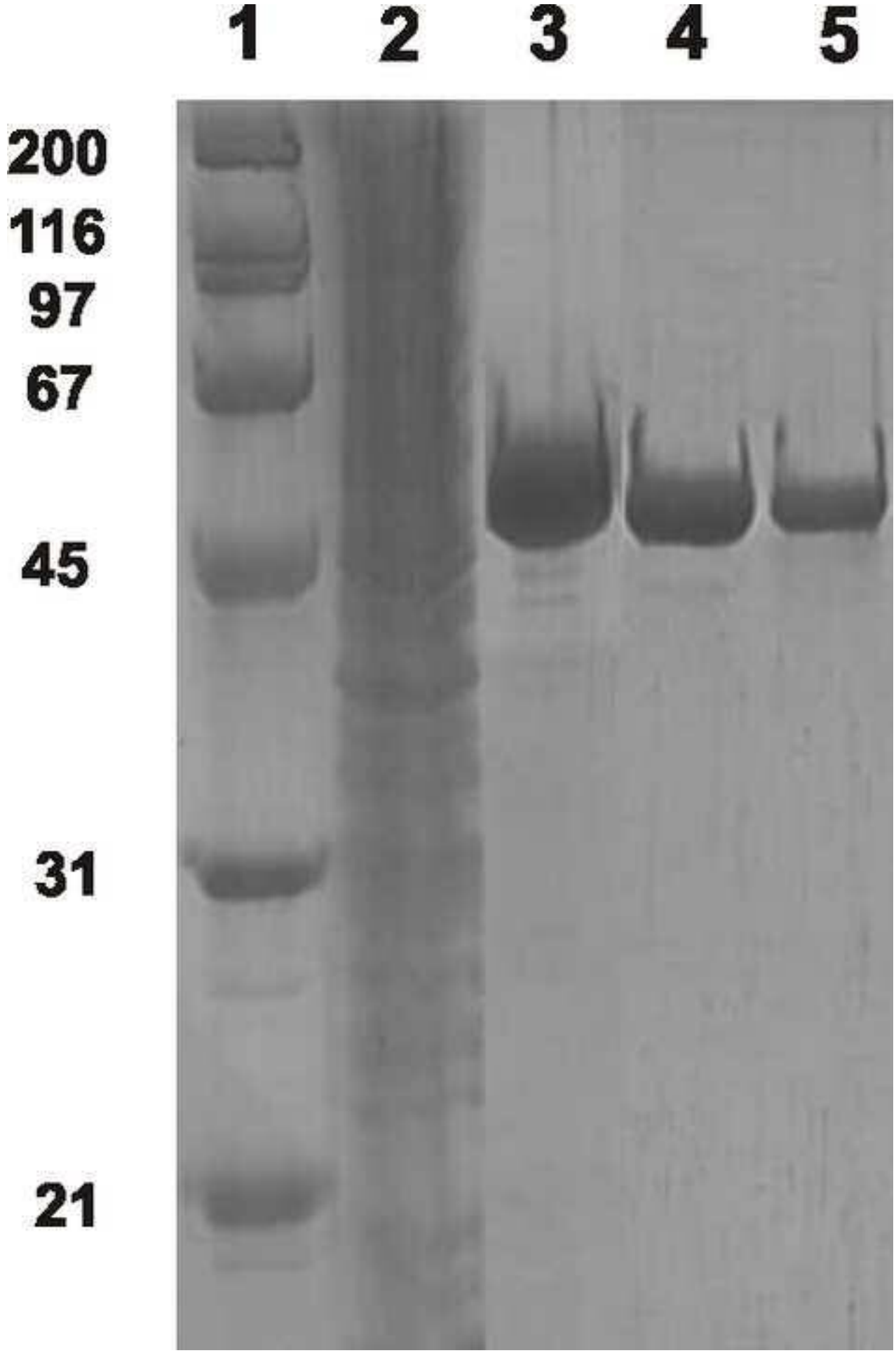

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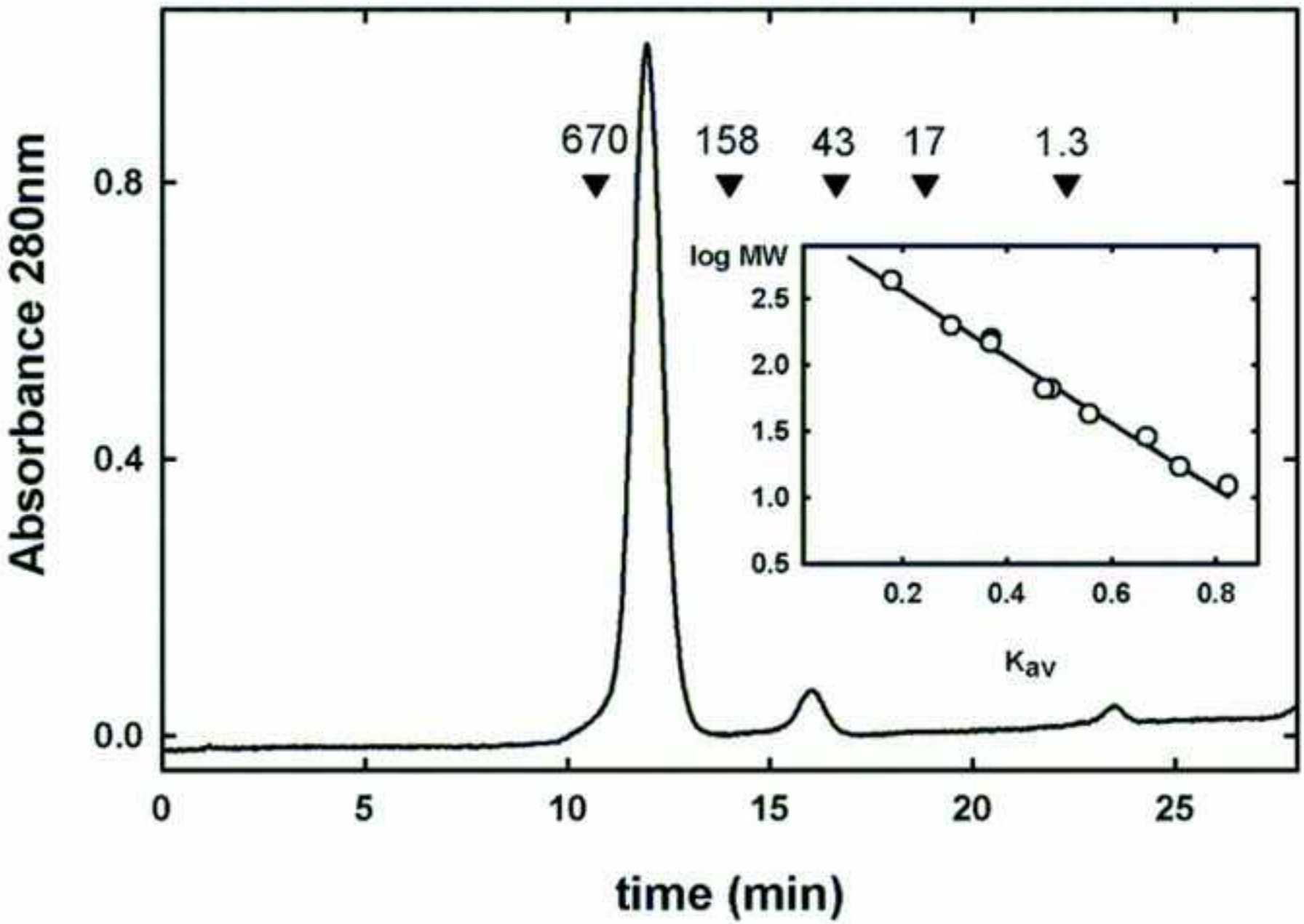

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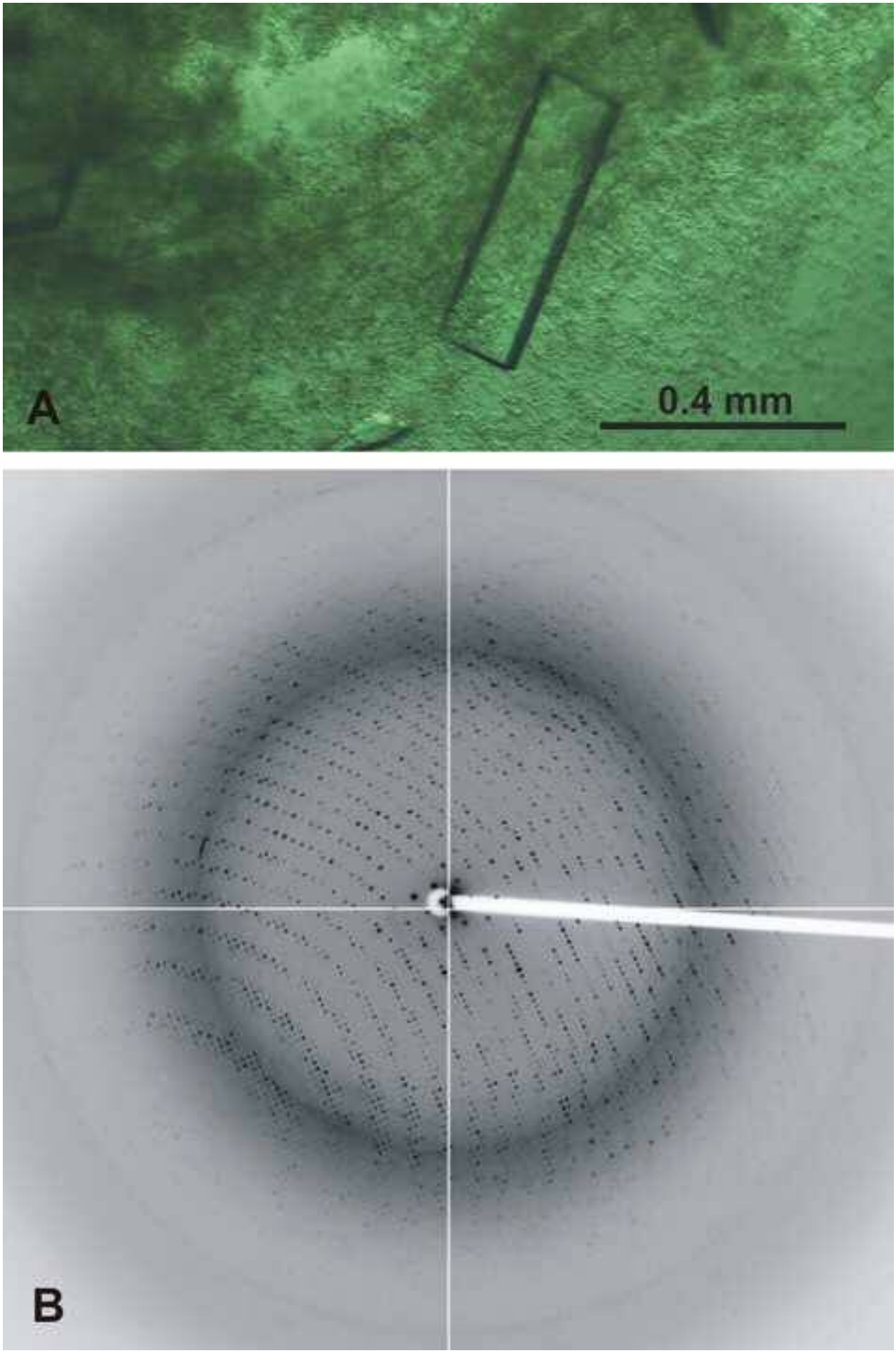

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