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ORIGINAL PAPER

A New Member of Family 11 Polysaccharide Lyase, Rhamnogalacturonan Lyase (*Ct*RGL*f*) from *Clostridium thermocellum*

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Abstract A thermostable, alkaline rhamnogalacturonan lyase (RG lyase) *Ct*RGL*f*, of family 11 polysaccharide lyase from *Clostridium thermocellum* was cloned, expressed, purified and biochemically characterised. Both, the full-length *Ct*RGL*f* (80 kDa) protein and its truncated derivative *Ct*RGL (63.9 kDa) were expressed as soluble proteins and displayed maximum activity against rhamnogalacturonan I (RG I). *Ct*RGL*f* showed maximum activity at 70 °C, while *Ct*RGL at 60 °C. Both enzymes showed maximum activity at pH 8.5. *Ct*RGL*f* and *Ct*RGL do not show higher activity on substrates with high β-D-galactopyranose (D-Gal*p*) substitution, this catalytic property deviates from that of some earlier characterised RG lyases which prefer substrates with high D-Gal*p* substitution. The enzyme activity of *Ct*RGL*f* and *Ct*RGL was enhanced by 1.5 and 1.3 fold, respectively, in the presence of 3 mM of Ca^{2+} ions. The TLC analysis of the degraded products of RG I, released by the action of *Ct*RGL*f* and *Ct*RGL revealed the production of RG oligosaccharides as major products confirming their endolytic activity.

Keywords Rhamnogalacturonan Lyase · Rhamnogalacturonan · Polysaccharide lyase · Pectin · *Clostridium thermocellum* · Rhamnogalacturonan oligosaccharides · Pectic oligosaccharides

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Introduction

The major components of plant cell wall are the polysaccharides that assemble to form a network. These polysaccharides are mainly cellulose, hemicellulose, and pectin. The plant cell wall is able to fulfil its structural role by providing strength and protection to the cell due to the presence of cellulose and hemicellulose [1]. The plant cell wall also has a functional role in cell–cell adhesion, cell signalling, wall porosity, pollen tube growth, leaf abscission and the pectin component of cell wall has been credited to play these roles [2]. Structural role of pectin in promoting upright growth of plants has also been reported [3]. Pectin is predominantly localised in the primary cell wall of all higher plants, gymnosperms, pteridophytes and bryophytes [4]. The principal components of pectin are homogalacturonan (HG) and the substituted HG:

Rhamnogalacturonan I (RG I) and Rhamnogalacturonan II (RG II). Xylogalacturonans and Apiogalacturonans are other less abundant substituted HG [2]. Homogalacturonan is a homopolymer of α-(1 → 4) linked D-galactopyranosyluronic acid (D-Gal*p*A) residues which may be methylated at C-6. RG I is composed of a main chain of alternating L-rhamnopyranosyl (L-Rha*p*) and D-Gal*p*A residues. The repeating monomeric unit is a disaccharide [→4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1→]. 20–80 % of L-Rha*p* residues are substituted with individual, linear or branched chains of L-arabinofuranosyl (L-Ara*f*) and D-Gal*p*. The side chains of RG I may be substituted with L-fucose or D-glucuronic acid (GlcA) [4]. RG I has been demonstrated to be crucial for normal development of periderm in potato [5]. Transgenic potato plants that expressed RG I cleaving enzyme developed morphological abnormalities like swelling of periderm cells. RG II main chain contrary to the name is also composed of α-(1 → 4) linked D-Gal*p*A with L-Rha*p* residues as substitutions apart from 12 other different monosaccharide residues [4].

Degradation of plant cell wall makes a reservoir of nutrients available for recycling. Nature has bestowed a diverse group of microorganisms with enzymes to breakdown the plant cell wall polysaccharides [6, 7]. When pectin comes under microbial attack, both glycoside hydrolases and polysaccharide lyases are recruited. Glycoside hydrolases cleave the glycosidic bonds via an acid–base catalysis mechanism [9]. Polysaccharide lyases cleave their substrates via a β-elimination mechanism, generating in the residue at the non-reducing end of the product a double bond between C-4 and C-5 [10]. Glycoside hydrolases and polysaccharide lyases have been classified into different families based on sequence similarity [11]. Many of the plant cell wall polysaccharide degrading enzymes are modular in nature and have one or more specialised substrate-binding module(s) referred to as carbohydrate-binding module(s) (CBM) in addition to a catalytic module.

Enzymes that can bring about deconstruction of plant cell wall polysaccharides are of key importance in conversion of lignocellulosic biomass into bioethanol and in production of prebiotics [12, 13]. The pectinases are one such class of industrial enzymes that are used in paper mills, textile industries, coffee and tea fermentations, treatment of feedstock for biofuel production and recovery of valuable products of plant origin like essential oils [14]. Some organisms are better at cleaving the recalcitrant structural polysaccharides of plant cell wall than others especially the anaerobic microorganisms which probably under selection pressure from the environment have evolved to possess highly efficient enzyme systems [15]. *C. thermocellum* is an anaerobic, thermophilic bacterium which presented to the researchers a new meaning of enzyme modularity and concerted enzyme action when the multi-enzyme complex referred to as cellulosome was first observed in it [16]. The cellulosomal complex has also been reported from other microorganisms [17]. Several studies focussing on cellulase and hemicellulases from this bacterium, which are more efficient than those from fungal origin, have been undertaken for bioethanol production [18, 19]. Despite never disappointing researchers in their search for cellulases and hemicellulases, pectin degrading enzymes of *C. thermocellum* are still to be characterised. The members of family 11 polysaccharide lyase (PL11) characterised earlier are rhamnogalacturonan lyases (RG lyases) from *Bacillus subtilis, Clostridium cellulolyticum, Cellvibrio japonicus* and *Bacillus licheniformis* [6–8, 20]. In the present study, cloning, expression and biochemical characterisation of a putative cellulosomal, family 11 PL enzyme (*Ct*RGL*f*) has been reported. *Ct*RGL*f* comprises a RG Lyase catalytic module, designated as *Ct*RGL and an associated putative family 35 CBM (Fig. 1a). *Ct*RGL*f* endolytically cleaves RG I component of pectin. To our knowledge this is the first modular RG lyase reported from *C. thermocellum* and is a new family 11 polysaccharide lyase member.

Materials and Methods

Sequence Analysis of *Ct*RGL*f*

The amino acid sequence of *Ct*RGL*f* (locus tag Cthe_0246) from *C. thermocellum* was accessed from the CAZy database (http://www.cazy.org/). The sequence with GenBank accession number: ABN51485.1 is classified as a family 11 PL according to the classification developed by Lombard et al. [11]. The sequence was subjected to BLAST analysis. The 'PDB' database was chosen for BLAST (http://www.rcsb.org/pdb/home/home.do). Conserved Domains Database was referred to determine the expanse of domains (http://www.ncbi.nlm.nih.gov/cdd/). The subcellular localisation of *Ct*RGL*f* was predicted using PSORT server (http://psort.hgc.jp/form.html).

Genomic DNA, Vector and Bacterial Strains

The genomic DNA of *C. thermocellum* ATCC 27405 was procured from DSMZ, Germany. The pET28a (+) (Novagen) expression vector was used for cloning and expression of PCR products. *Escherichia coli* DH5α cells (Novagen) were used as host for amplification of recombinant plasmids. *E. coli* BL21 (DE3) (Novagen) and *E. coli* (pLysS) (DE3) (Novagen) were used as host for expression of proteins.

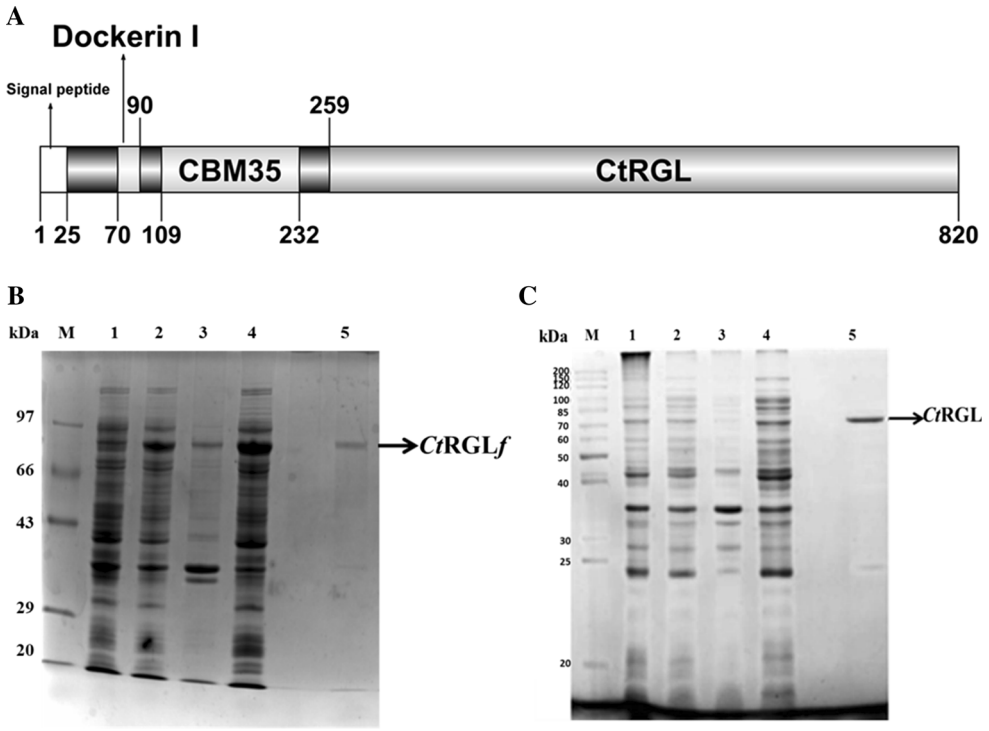

Fig. 1 **a** Molecular architecture of protein *Ct*RGL*f* showing modular structure with a CBM35 module towards the N-terminal connected to the catalytic module, *Ct*RGL via a linker sequence. **b** Expression and purification of *Ct*RGL*f* analysed on SDS-PAGE gel (12 %, w/v); lane M—Molecular mass marker (14.3–97.4 kDa, Genei), lane 1—uninduced *E. coli* (DE3) BL21 cells, lane 2—induced *E. coli* (DE3) BL21 cells, lane 3—cell pellet after sonication, lane 4—cell free extract, lane 5—purified protein and **c** Expression and purification of *Ct*RGL analysed on SDS-PAGE gel (12 %, w/v); lane M—molecular mass marker (10-200 kDa, Fermentas), lane 1—uninduced *E. coli* (DE3) pLysS cells, lane 2—induced *E. coli* (DE3) pLysS cells, lane 3—cell pellet after sonication, lane 4—cell free extract, lane 5—purified protein

Cloning of Full-Length *Ct*RGL*f* and Truncated Derivative *Ct*RGL

The nucleotide sequences of full-length family 11 PL from *C. thermocellum*, *Ct*RGL*f*, and its truncated derivative, *Ct*RGL were amplified using 0.025 U/µl of Taq DNA polymerase (Bioline, UK). The 50 µl PCR reactions contained 0.45 µM each of forward and reverse primers, 1.5 mM $MgCl_2$ and 2 mM dNTPs. The sequences of the forward primer and the reverse primer used for the amplification of gene encoding *Ct*RGL*f* were FP1: 5′-CGGCTAGCACGA GATATCAGGCTGAGG-3′ and RP1: 5′-CCCTCGAGT TACGGCACAAGGTAAATATTTGG-3′. The reverse primer for amplification of gene encoding *Ct*RGL was same as that for *Ct*RGL*f*, while the sequence of the forward primer was FP2: 5′-CGGCTAGCATGGAGAGCTGGACAGAG-3′. The forward and reverse primers contained *NheI* and *XhoI* restriction sites (underlined), respectively. The PCR conditions used were initial denaturation at 94 °C for 4 min and 94 °C for 30 s, 49 °C for 60 s, 72 °C for 60 s for a total of 30 cycles followed by final extension at 72 °C for 10 min on a thermal cycler (TAKARA Bio, Japan). The PCR products were separated on 0.8 % (w/v) agarose gel. The DNA fragments encoding *Ct*RGL*f* (2136 bp) and *Ct*RGL (1686 bp) were excised from the gel and purified using GenElute kit (Sigma Chemical Company, USA). The purified PCR products and pET28a (+) expression vector were digested with *NheI* and *XhoI* restriction enzymes (Promega, USA). The restriction enzymes digested PCR products and the vector were ligated by T4 DNA ligase (Promega, USA). The ligation reaction mixtures were transformed into *E. coli* DH5α cells for plasmid DNA amplification. The positive clones were confirmed by restriction digestion of the isolated plasmid DNA.

Expression of Recombinant Proteins and Culture Conditions

E. coli BL21 (DE3) cells were used as host for expression of *Ct*RGL*f* and were aerobically cultured in 600 ml LB medium supplemented with kanamycin (50 µg/ml) at 37 °C, 180 rpm. *Ct*RGL was expressed in *E. coli* BL21 (DE3) pLysS cells, which were aerobically cultured in 600 ml Terrific Broth supplemented with chloramphenicol (34 µg/ml) and kanamycin (50 µg/ml) incubated at 37 °C and 180 rpm [21]. After the cell growth reached mid

exponential phase ($A_{600} = 0.6$), the cells were cooled to 16 °C and then isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM and further incubated at 16 °C and 180 rpm for 20 h.

Purification of Recombinant Proteins

The *E. coli* cells (600 ml culture) harbouring the plasmids containing genes encoding *Ct*RGL*f* and *Ct*RGL after IPTG induction were harvested by centrifugation at 9000×*g*, 4 °C for 10 min. The cell pellet was washed with 50 mM Tris–HCl buffer (pH 8.5) containing 100 mM NaCl and 50 mM imidazole, re-suspended in the same buffer. The re-suspended cells were subjected to ultra-sonication (Sonics, Vibra cell) for 8–12 min (5 s on and 15 s off pulse; 33 % amplitude), and the lysed cells were centrifuged at 20,000×*g*, 4 °C for 50 min. The supernatant (cell free extract) obtained after centrifugation was filtered through 0.45 μm membrane and applied to 1 ml Ni^{2+} ion chelating immobilised ion affinity chromatography (IMAC) column (HiTrap, GE Healthcare, USA). The column was pre-equilibrated with 50 mM Tris–HCl buffer (pH 8.5) containing 100 mM NaCl and 50 mM imidazole. Both *Ct*RGL*f* and *Ct*RGL contained a stretch of six histidine residue tag at N-terminal and were eluted with a linear gradient of imidazole (0–500 mM) in 50 mM of Tris–HCl buffer (pH 8.5) containing 100 mM NaCl. The purified *Ct*RGL*f* and *Ct*RGL enzymes were dialysed against 50 mM Tris–HCl buffer (pH 8.5) with several changes.

Enzyme Activity Assay

One ml reaction mixture containing 0.15 % (w/v) substrate dissolved in 50 mM of Tris–HCl buffer (pH 8.5) and 3 mM $CaCl_2$ and the recombinant enzyme was incubated at 60 °C for 5 min. After incubation, the reaction mixture was kept on ice for 5 min to stop the reaction and then centrifuged at 13,000×*g* and the supernatant was used to measure the absorbance. The enzyme activity was assayed by monitoring the formation of Δ4,5 unsaturated D-Gal*p*A residue at the non-reducing end of the cleaved substrate by measuring its absorbance at 235 nm (A_{235}) on a UV–Visible spectrophotometer (Gene-Quant, GE Healthcare, USA). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of product per minute. The enzyme activity was calculated using the known molar coefficient of 4600 M^{-1} for Δ4,5 unsaturated D-Gal*p*A [22].

Substrate Specificity

The substrate specificity of *Ct*RGL*f* and *Ct*RGL was investigated using various pectic substrates (1 %, w/v) viz. rhamnogalacturonan from soybean (RGS), rhamnogalacturonan from potato (RGP), pectic galactan from lupin (PGL), galactan from potato (GP), polygalacturonic acid (PGA) and pectin (25 % methyl-esterified) under optimised conditions. *Ct*RGL*f* or *Ct*RGL was incubated with substrate in 50 mM Tris–HCl buffer (pH 8.5) containing 3 mM $CaCl_2$ for 5 min at optimum temperature and the A_{235} was recorded as mentioned in previous section. RGS, RGP, PGL and GP were purchased from Megazyme, Ireland. PGA and pectin (25 % methyl-esterified) were purchased from Sigma-Aldrich Corporation, USA.

Biochemical Characterisation

The optimum pH for the activity *Ct*RGL*f* or *Ct*RGL was determined by incubating the enzymes with 0.15 % (w/v) RGS at 60 °C for 5 min in different buffers containing 3 mM $CaCl_2$: 50 mM MES (pH 6.0), 50 mM Tris–HCl (pH 7.0–9.0), 50 mM Glycine-NaOH (pH 10.0) and 50 mM CAPS (pH 11.0). The A_{235} was measured and the enzyme activity was calculated. To determine the optimum temperature for the activity of *Ct*RGL*f* or *Ct*RGL, the enzymes were incubated at different temperatures ranging from 30 to 100 °C in 50 mM Tris–HCl buffer (pH 8.5) containing 0.15 % (w/v) RGS and 3 mM $CaCl_2$. The A_{235} was measured and the enzyme activity was calculated as described earlier. The effect of temperature on stability of *Ct*RGL*f* and *Ct*RGL was determined by incubating the enzymes in Tris–HCl buffer (pH 8.5) at different temperatures ranging from 30 to 100 °C for 30 min followed by determination of activity at respective optimum temperatures. The kinetic parameters of *Ct*RGL*f* and *Ct*RGL were determined by assaying their activity against different concentrations of substrates under the optimised conditions of temperature and pH. The effect of various metal ions on the activity of *Ct*RGL*f* or *Ct*RGL was determined by pre-treating them with 10 mM EDTA at 25 °C for 60 min to remove any bound divalent metal ion, followed by buffer exchange to 50 mM Tris–HCl (pH 8.5). The activity was measured in the presence of $CaCl_2$, $MgCl_2$, $MnCl_2$ or $CoCl_2$ at a final concentration of 3 mM. The effect of Ca^{2+} ions on the activity of *Ct*RGL*f* and *Ct*RGL was determined by varying the $CaCl_2$ concentration from 0 to 8 mM in the reaction mixture.

Thin-Layer Chromatography Analysis of *Ct*RGL*f*- and *Ct*RGL-Treated RGS

The mode of action of *Ct*RGL*f* and *Ct*RGL on RGS was determined by analysing the degradation products formed at different time intervals using thin-layer chromatography. Separate reaction mixtures (1 ml) of *Ct*RGL*f* and *Ct*RGL were set that contained 0.15 % (w/v) RGS and 3 mM

$CaCl_2$ in 50 mM Tris–HCl buffer (pH 8.5). A separate reaction was set for each time interval (0, 5, 15, 30 min, 1, 12 and 24 h). The large undigested polysaccharide molecules were precipitated by adding 2 volumes of absolute ethanol followed by centrifugation at 13,000×*g* for 10 min. The supernatant (1 ml) containing enzyme-degraded products of RGS was collected in separate microcentrifuge tubes and concentrated to 20 µl by incubating at 60 °C for 16 h. The concentrated sample (1 µl) was applied as a spot on a TLC plate (Merck, Germany) that was later developed in a solution containing 1-butanol, acetic acid and water in 5:2:3 ratio. The TLC plates were stained by diphenylamine-aniline-phosphoric acid reagent (1 ml 37.5 % HCl, 2 ml aniline, 10 ml 85 % H_3PO_4, 100 ml ethyl acetate and 2 g diphenylamine) and visualised after incubating at 80 °C for 30 min [23].

Results and Discussion

Sequence Analysis of *Ct*RGL*f*

The amino acid sequence with locus tag Cthe_0246 from *C. thermocellum* in the CAZy database was found to be a part of 2461 bp long ORF. A prokaryotic ribosome-binding site (GGAGG) was identified 8 bp upstream of the putative translational start codon. BLAST analysis revealed the modular structure of the protein represented by the amino acid sequence in the form of three distinct domains. The N-terminal cleavage site of the signal peptide was predicted between Ala25 and Gly26 by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP-3.0/), which was same as that predicted using rules of Perlman and Halvorson (1983) [24]. Towards the N-terminal, downstream of the signal peptide a stretch of 20 amino acids showed similarity to Dockerin I from *C. thermocellum* (Fig. 1a). The second module spanning from amino acids residue 109–232 was identified to be a putative family 35 CBM as it showed similarity to an already characterised CBMs from *C. thermocellum*. Towards C-terminal, the catalytic module ranging from amino acid residue 259–820 exhibited 62 and 59 % sequence identity with two RG Lyases YesW and YesX, respectively, from *B. subtilis* [6]. A schematic representation of the molecular architecture of *Ct*RGL*f* presented in Fig. 1a shows that the N-terminal putative Dockerin I module is connected to the putative CBM35 module which is in turn connected to a (RG lyase module (catalytic domain) via a small linker sequences. The interaction between enzymes borne Dockerin modules and the Cohesin modules of a scaffold protein gives rise to the cellulosomal complex [15]. The presence of a putative Dockerin I at N-terminal and the subcellular localisation score predicted by PSORT server indicated that the protein encoded by sequence Cthe_0246 (*Ct*RGL*f* is a derivative of Cthe_0246 without the N-terminal Dockerin I) is an extracellular enzyme and probably integrates as a component of the *C. thermocellum* cellulosome.

Purification of Recombinant *Ct*RGL*f* and *Ct*RGL

The recombinant *Ct*RGL*f* and *Ct*RGL expressed as soluble proteins and after purification displayed homogeneous single bands on 12 % SDS-PAGE gels (Fig. 1b, c). The molecular masses of the recombinant *Ct*RGL*f* and *Ct*RGL including the N-terminal histidine tag were calculated to be 80.2 and 63.9 kDa, respectively, which are in close agreement with those observed on SDS-PAGE gels. The purification folds of *Ct*RGL*f* and *Ct*RGL were 21 and 37, respectively. The activity yields of *Ct*RGL*f* and *Ct*RGL were 17 and 1.8 %, respectively (Table 1). *Ct*RGL showed a tendency to form inclusion bodies resulting in a low activity yield which was also reported for RG lyase, Rgl11Y from *C. cellulolyticum* [7].

Substrate Specificity of *Ct*RGL*f* and *Ct*RGL

The β-elimination reaction mechanism of *Ct*RGL*f* and *Ct*RGL was confirmed by the increase in the absorbance at 235 nm, when the enzymes were incubated with their substrate RGS, which is due to the formation of double bond in the D-Gal*p*A residue at the newly formed non-reducing end. Both *Ct*RGL*f* and *Ct*RGL displayed high activity against two RGs (RGS and RGP) and moderate activity against pectic galactan (PGL). Both the enzymes showed low activity against PGA and pectin (25 % methyl-esterified) (Table 2). *Ct*RGL*f* and *Ct*RGL were primarily active against rhamnogalacturonans but they could also cleave the homogalacturonan component of pectin. It may be easy to comprehend that the low activities of *Ct*RGL*f* and *Ct*RGL against PGL may be due to the low content of L-Rha*p* and D-Gal*p*A residues present in this substrate when compared to both RGs (RGS and RGP). However, it is noteworthy that this catalytic property of *Ct*RGL*f* and *Ct*RGL is in stark contrast to that of some other RG lyases, Rgl11Y and Rgl11A from *C. cellulolyticum* and *P. cellulosa,* respectively [7, 8]. Rgl11A and Rgl11Y displayed higher activity on PGP, despite its low L-Rha*p* and D-Gal*p*A contents. This highlights the role of β-D-galactopyranose (D-Gal*p*) substitutions for their enhanced activity on PGP as it is highly substituted with D-Gal*p* residues [7, 8]. All these substrates contain RG I main chain, but differ in the percentage of D-Gal*p* residues in their side chains. PGL and PGP contain around 74 and 87 % D-Gal*p*, respectively, while RGP and RGS contain only 20 % D-Gal*p*. Very recently, another RG lyase belonging to PL family 4, PcRGL4A from *Penicillium*

Table 1 Production and purification of *Ct*RGL*f* and *Ct*RGL

Purification step	Volume (ml)	Activity*		Protein			Specific activity* (U/mg)	Purification fold
		U/ml	Total activity(U)	Activity yield (%)	(mg/ml)	Total protein (mg)		
*Ct*RGL*f*								
Cell free extract	25	3.8	95	–	8.7	217	0.43	1
IMAC	8	2	16	17	0.22	1.76	9	21
*Ct*RGL								
Cell free extract	25	3.1	79	–	26	650	0.12	1
IMAC	1.5	1	1.5	1.8	0.22	0.34	4.5	37

* Enzyme activity was measured using RGS (1 %, w/v) as substrate dissolved in 50 mM Tris–HCl buffer (pH 8.5) containing 3 mM $CaCl_2$, under optimised conditions as mentioned in "Materials and Methods" section

Table 2 Substrate specificity and kinetic parameters of *Ct*RGL*f* and *Ct*RGL

Substrate (1 %, w/v)	*Ct*RGL*f*				*Ct*RGL			
	Specific Activity (U/mg)	K_m (mg/ml)	k_{cat} (s^{-1})	k_{cat}/K_m (ml/mg/s)	Specific Activity (U/mg)	K_m (mg/ml)	k_{cat} (s^{-1})	k_{cat}/K_m (ml/mg/s)
Rhamnogalacturonan (Soybean)	9.8	4.8	21.2	4.41	5.8	5.1	6.9	1.35
Rhamnogalacturonan (Potato)	9.1	4.9	21.0	4.28	5.4	5.3	6.0	1.13
Pectic galactan (Lupin)	4.7	10	7.7	0.77	3.0	11.2	4.8	0.43
Galactan (Potato)	3.8	4.7	6.4	1.36	1.2	6.4	2.1	0.32
Pectin (25 % methyl-esterified)	4.3	8.2	9.8	1.19	1.3	7.6	2.3	0.3
Polygalacturonic acid	1.2	ND	ND	ND	1.2	ND	ND	ND

The reaction of *Ct*RGL*f* and *Ct*RGL was carried out at 70 °C and 60 °C, respectively, for 5 min using pectic substrates (1 %, w/v) dissolved in 50 mM Tris–HCl buffer (pH 8.5) containing 3 mM $CaCl_2$

ND not determined

chrysogenum has been reported which too does not preferentially degrades substrates with higher D-Gal*p* substitution [25].

Biochemical Properties of *Ct*RGL*f* and *Ct*RGL

The optimum temperature for the activity of *Ct*RGL*f* was 70 °C, whereas for *Ct*RGL it was 60 °C (Fig. 2a). Optimum pH for the activity of both *Ct*RGL*f* and *Ct*RGL was 8.5 (Fig. 2b), similar to other RG lyases from *B. subtilis, C. cellulolyticum, C. japonicus and B. licheniformis* [6–8, 20]. *Ct*RGL*f* was more thermostable than *Ct*RGL as it retained 90 % of its activity after incubation at 60 °C for 30 min, whereas *Ct*RGL showed only 45 % of its activity obtained under optimised assay conditions (Fig. 2c). The higher thermal stability of *Ct*RGL*f* may be attributed to the presence of CBM35. A Family 13 CBM has also been recently reported to provide thermostability to its associate catalytic module [26]. The K_m values of *Ct*RGL*f* and *Ct*RGL with RGS were similar, 4.8 mg/ml and 5.1 mg/ml, respectively (Table 2). This indicated that *Ct*RGL, the truncated form of *Ct*RGL*f* can cleave RGS, independently even in the absence of CBM. The k_{cat} values of *Ct*RGL*f* and *Ct*RGL against RGS were 21 and 7 s^{-1}, respectively (Table 2). The lower k_{cat} value of *Ct*RGL may be attributed to the low activity yield obtained (Table 1) which might have been due to the formation of inclusion bodies as mentioned in "Purification of recombinant *CtRGLf* and *CtRGL*" section. Structural determination of RG lyases from *B. subtilis* established an important role of calcium ions for enzyme catalysis [27]. The sequence analysis of *Ct*RGL*f* revealed the presence of conserved Ca^{2+} ions binding amino acid residues. Therefore, the effect of divalent metal ions on the activity of *Ct*RGL*f* and *Ct*RGL was analysed after the treatment with 10 mM EDTA which completely abolished the enzyme activity (Table 3). These results highlighted the important role of divalent metal ions on the activity of *Ct*RGL*f* and *Ct*RGL. In the absence of any metal ion *Ct*RGL*f* and *Ct*RGL showed 6.5 and 4.4 U/mg, respectively, which were 67 and

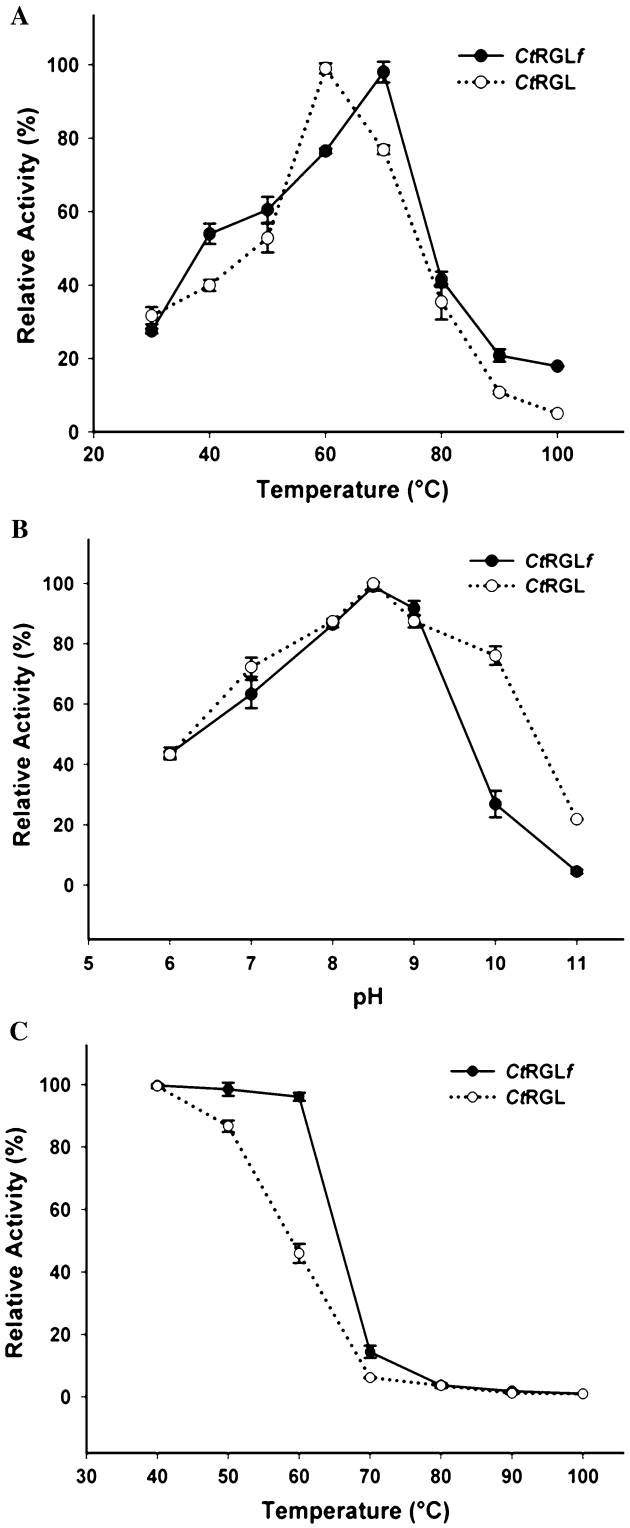

Fig. 2 **a** Effect of temperature on activity of *Ct*RGL*f* and *Ct*RGL. The activity at 70 °C for *Ct*RGL*f* and activity at 60 °C for *Ct*RGL was taken as 100 %, **b** effect of pH on activity of *Ct*RGL*f* and *Ct*RGL. The activities of both *Ct*RGL*f* and *Ct*RGL at pH 8.5 were taken as 100 %, **c** thermal stability of *Ct*RGL*f* and *Ct*RGL. The activity of the enzymes incubated at 4 °C was taken as 100 %

77 %, respectively, of their activities in the presence of 3 mM Ca^{2+} ions. Among the various metal ions tested, Ca^{2+} ions (3 mM) were able to restore the enzyme activity of EDTA-treated *Ct*RGL*f* and *Ct*RGL up to 45 and 35 %, respectively, when compared with the activity of EDTA-untreated enzymes in the presence of 3 mM Ca^{2+} (Table 3). However, Mn^{2+} ions (3 mM) resulted in higher reactivation than Ca^{2+} ions (3 mM), restoring the enzyme activity of EDTA-treated *Ct*RGL*f* and *Ct*RGL up to 96 and 77 %, respectively. Ochiai et al. have also reported that Mn^{2+} ions were more potent than Ca^{2+} ions in restoring the activity of EDTA-treated two RG lyases from *B. subtilis* [6].

Two different types of RG lyases have been reported, the first which do not have an absolute requirement of calcium ions and show optimum activity at acidic pH and the second which requires calcium ions and show optimum activity at basic pH. RG lyase from *Erwinia chrysanthemi* is active in acidic pH and does not depend on Ca^{2+} ions for its activity [28]. RG lyases from *B. subtilis*, *C. cellulolyticum*, *C. japonicas* and *B. licheniformis* are active in alkaline pH and require Ca^{2+} ions [6, 7, 20]. *Ct*RGL*f* and *Ct*RGL were optimally active at alkaline pH and required Ca^{2+} ions for their activity (Fig. 3). The activities of *Ct*RGL*f* and *Ct*RGL were enhanced by 1.5 and 1.3 fold, respectively, by 3 mM Ca^{2+} ions.

Thin-Layer Chromatography Analysis of *Ct*RG*Lf*- and *Ct*RGL-Treated RGS

The profiles of time-dependent enzymatic degradation of RGS by *Ct*RGL*f* and *Ct*RGL, analysed by TLC, are shown in Fig. 4. It has been reported earlier that the smallest

Table 3 Effect of metal ions on *Ct*RGL*f* and *Ct*RGL activities

Pre-treatment	Metal ion (3 mM)	Activity (%)	
		*Ct*RGL*f*	*Ct*RGL
No EDTA	–	67	77
	*Ca^{2+}	100	100
	Mn^{2+}	100	70
	Mg^{2+}	81	76
	Co^{2+}	14	14
EDTA (10 mM)	–	0	0
	Ca^{2+}	45	35
	Mn^{2+}	96	77
	Mg^{2+}	32	25
	Co^{2+}	48	29

* Specific activities of *CtRGLf* and *CtRGL* in the presence of 3 mM Ca^{2+} ions were 9.8 and 5.8 U/mg, respectively

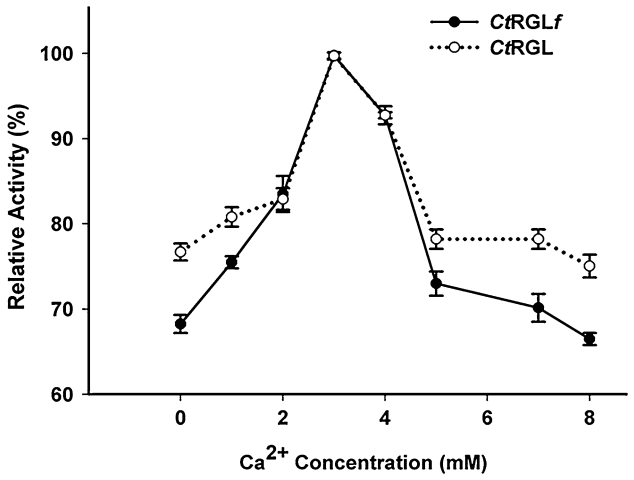

Fig. 3 Effect of Ca^{2+} ions ($CaCl_2$) on the activities of *Ct*RGL*f* and *Ct*RGL. The activity of both enzymes at 3 mM Ca^{2+} ions concentration was taken as 100 %

possible reaction product generated by the activity of a RG lyase on RG I is unsaturated RG disaccharide whose mobility is similar to that of D-Gal*p*A [6]. All the samples of both *Ct*RGL*f* and *Ct*RGL collected at different time periods over 24 h displayed the products of variable sizes, appearing as discrete spots with much less mobility than D-Gal*p*A on the TLC plate. This suggested that both *Ct*RGL*f* and *Ct*RGL cleave the substrate endolytically and produced oligosaccharides larger than the unsaturated RG disaccharide as the major products. This type of substrate cleavage pattern was also displayed by an endo- RG lyase (YesW) from *B. subtilis* [6]. ATP-binding cassette (ABC) transporters are trans-membrane proteins involved in metabolite translocation across the cell membrane [29]. Owing to the presence of the gene encoding a putative ABC transporter downstream of the ORF encoding *Ct*RGL*f*, it may be speculated that the oligosaccharides produced by *Ct*RGL*f* are taken up by *C. thermocellum* cells. However, till date, the existence of any pathway or enzymes which might metabolise these oligosaccharides is not known for *C. thermocellum.* The pectin and pectic oligosaccharides have been reported to show anti-proliferative effects on the human colonic adenocarcinoma cell line HT29 [30]. The RGI component of potato was reported to inhibit the proliferation of HT29 colon cancer cells [31]. The RG oligosaccharides produced by action of *Ct*RGL*f* may be studied for therapeutic applications. Besides reporting a novel thermophilic RG lyase, this study presents cues to the potential of *C. thermocellum* to degrade RGI component of pectin and reinforces its versatility as a degrader of the plant biomass which is critical for recycling the nutrients in the ecosystem.

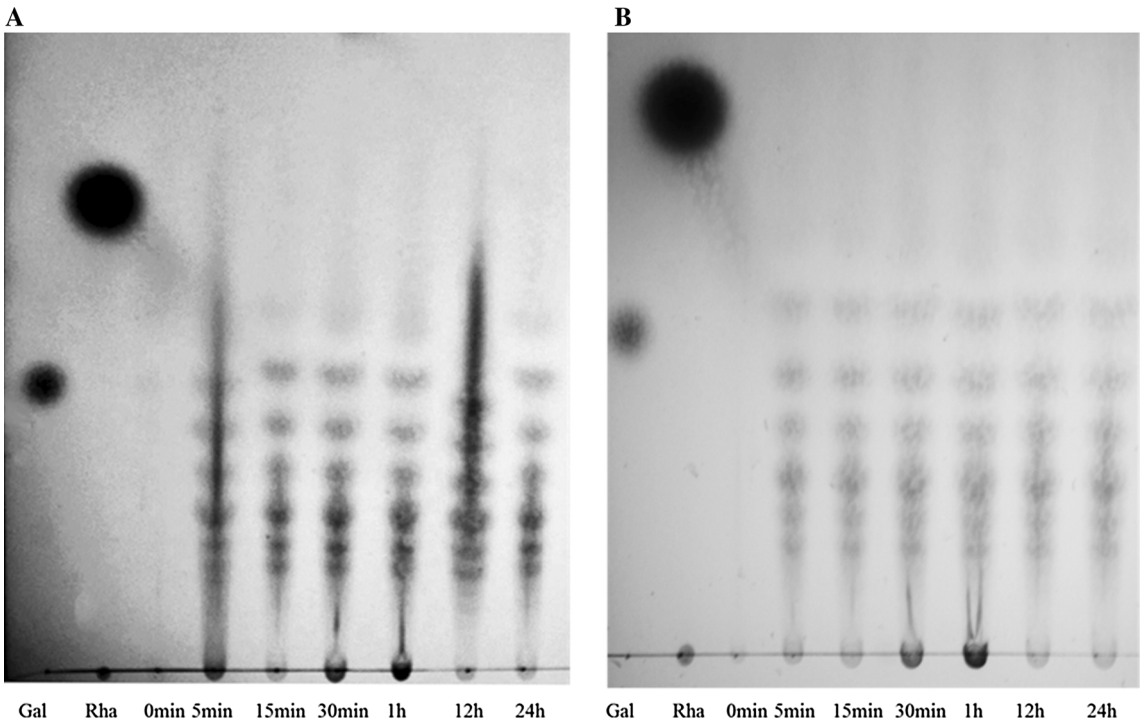

Fig. 4 TLC analysis of degradation products of **a** *Ct*RGL*f* and **b** *Ct*RGL. Gal- β-Gal*p*A and Rha- α-Rha*p* were used as standards

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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