Binding and structural studies of the complexes of type 1 ribosome inactivating protein from *Momordica balsamina* with cytosine, cytidine, and cytidine diphosphate

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**A B S T R A C T**

The type 1 ribosome inactivating protein from *Momordica balsamina* (MbRIP1) has been shown to interact with purine bases, adenine and guanine of RNA/DNA. We report here the binding and structural studies of MbRIP1 with a pyrimidine base, cytosine; cytosine containing nucleoside, cytidine; and cytosine containing nucleotide, cytidine diphosphate. All three compounds bound to MbRIP1 at the active site with dissociation constants of $10^{-4}$ M to $10^{-7}$ M. As reported earlier, in the structure of native MbRIP1, there are 10 water molecules in the substrate binding site. Upon binding of cytosine to MbRIP1, four water molecules were dislodged from the substrate binding site while five water molecules were dislodged when cytidine bound to MbRIP1. Seven water molecules were dislocated when cytidine diphosphate bound to MbRIP1. This showed that cytidine diphosphate occupied a larger space in the substrate binding site enhancing the buried surface area thus making it a relatively better inhibitor of MbRIP1 as compared to cytosine and cytidine. The key residues involved in the recognition of cytosine, cytidine and cytidine diphosphate were Ile71, Glu85, Tyr111 and Arg163. The orientation of cytosine in the cleft is different from that of adenine or guanine indicating a notable difference in the modes of binding of purine and pyrimidine bases. Since adenine containing nucleosides/nucleotides are suitable substrates, the cytosine containing nucleosides/nucleotides may act as inhibitors.

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1. Introduction

The ribosome inactivating proteins (RIPs) are N-glycosidases which are known to remove the specific purine residues from the sarcin/ricin (S/R) loop of the large rRNA [1,2]. This leads to the inhibition of protein synthesis in the cell [3]. The X-ray crystallographic studies have shown that RIPs bind specifically to adenine base [4]. A number of structures of type 1 RIPs from different sources in native forms [5–15] as well as of its several complexes with various ligands are already known [3,12,13]. The type 1 RIPs catalyze the removal of adenine residue from the sarcin/ricin loop (SRL) of rRNA in eukaryotic ribosomes [2,3]. These RIPs have also been shown to recognize the mRNA 5’ cap structures with m7GpppN motif where N is any base [12]. There are several reports about interactions of RIPs with purine bases of RNA such as adenine and guanine but no structural information is available on the mode of binding of pyrimidine bases such as cytosine and thymine. Therefore, it will be of interest to examine the modes of binding of pyrimidine bases to a type 1 RIP. We report here the binding studies of a type 1 RIP from *Momordica balsamina* (MbRIP1) with three compounds, (i) cytosine (CYS), (ii) cytidine (CYT), and (iii) cytidine diphosphate (CDP). All three compounds bound to MbRIP1 at the active site with dissociation constants of $10^{-4}$ M to $10^{-7}$ M. The orientation of cytosine in the substrate binding cleft is found to be different from that of adenine or guanine indicating a notable difference in the modes of binding of purine and pyrimidine bases. Since adenine containing nucleosides/nucleotides are suitable substrates, the cytosine containing nucleosides/nucleotides may act as inhibitors.

2. Materials and methods

2.1. Isolation and purification

The purification of MbRIP1 was carried out using the protocol reported earlier [13]. The samples of purified protein were concentrated using Amicon ultrafiltration device (Millipore Corporation, Bedford, USA) with a membrane having a molecular weight

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at a concentration of 1 mM. The concentration of all the three ligands were varied by adding increasing volumes of 1 mM, 2 mM and 4 mM of sodium phosphate buffer at pH 6.8 at concentrations of 25 mM sodium phosphate buffer pH 6.8. The stock solutions of CYS, CYT and CDP were also prepared in 25 mM of sodium phosphate buffer at pH 6.8 at a concentration of 1 mM. The concentration of CYS, CYT and CDP respectively from their stock solutions made cut off of 3 kDa.

2.2. Binding studies using fluorescence

The binding studies of MbRIP1 with compounds (i) CYS, (ii) CYT and (iii) CDP were carried out with spectrofluorometer FP-6200 (Shimadzu, Kyoto, Japan). 2.5 mg of protein was dissolved in 25 mM sodium phosphate buffer pH 6.8 at concentrations of 1 μM, 2 μM, 5 μM, 10 μM, and 20 μM of CYS, CYT and CDP respectively from their stock solutions made at a concentration of 1 mM. The fluorescence experiments were conducted under the conditions of both entrance and exit slit widths at 5 nm and scanning speed of 240 nm/min. The fluorescence spectra of protein were recorded in the range of 300–400 nm at an excitation of 280 nm at 298 K (Fig. 1). All the observations were repeated six times for estimating the experimental errors. The spectral changes of the protein were recorded with different ligands at various concentrations.

![Fig. 1. Binding curves for cytosine (1), cytidine (2), and cytidine diphosphate (3) showing the changes in fluorescence intensities (ΔF/F0) at 280 nm as the three compounds bound to MbRIP1 with increasing molar concentrations of ligands. The errors on the experimental points have been indicated.](image)

**Table 1**

| Parameter                  | CYS | CYT | CDP |
|----------------------------|-----|-----|-----|
| PDB ID                     | 4ZT8| 5CSO| 5CST|
| Space group                | H3  | H3  | H3  |
| Unit cell dimensions (Å)   | a=b | c   | 9   |
| Resolution range (Å)       | 37.63±1.98 | 37.62±1.78 | 38.11±1.78 |
| Total number of reflections| 83496| 149580| 132103|
| Number of unique reflections| 16671| 22979| 22958|
| Overall completeness (%)   | 99.9(99.5) | 99.8(96.9) |
| Overall R㎡(%)             | 7.3(28.3) | 5.0(27.2) | 6.5(49.2) |
| Overall R(%)               | 32.3(4.2) | 51.2(4.8) | 34.0(2.6) |
| Fluorescence measurements  | 20.4 | 21.4 | 18.9 |
| Protein atoms              | 1911 | 1911 | 1911 |
| Ligand atoms (one molecule)| 8 (CYS) | 17(CYT) | 25 (CDP) |
| Carbohydrate atoms (NAG)   | 14  | 14  | 14  |
| Water molecules            | 220 | 246 | 245 |
| R.m.s.d in bond lengths (Å)| 0.01| 0.01| 0.02 |
| R.m.s.d in bond angles (deg)| 1.08| 1.09| 2.0 |
| R.m.s.d in dihedral angles (deg)| 13.1| 12.4| 12.3 |
| Overall G factor           | 0.14| 0.15| –0.02|
| Wilson B-factor (Å²)       | 24.1| 23.9| 21.8 |
| Mean B-factor for main chain atoms | 28.7 | 28.2 | 25.7 |
| Mean B-factor for side chain atoms and water oxygen atoms | 32.5 | 33.4 | 33.1 |
| Mean B-factor for all atoms| 30.8| 31.1| 29.7 |
| Residues in the most favored regions (%) | 93.2 | 92.7 | 93.2 |
| Residues in the additionally allowed regions (%) | 6.4 | 6.8 | 6.4 |
| Residues in the disallowed regions (%) (Leu 77 as part of type II β-turn) | 0.5 | 0.5 | 0.5 |

The values in parentheses correspond to the values in the highest resolution shell.

**Table 2**

| Parameter          | CYS | CYT | CDP |
|--------------------|-----|-----|-----|
| Resolution range (Å) | 37.63±1.98 | 37.62±1.78 | 38.11±1.78 |
| Total reflections   | 83496| 149580| 132103|
| Number of reflections| 16671| 22979| 22958|
| Overall completeness (%) | 99.9(99.5) | 99.8(96.9) |
| Overall R㎡(%)     | 7.3(28.3) | 5.0(27.2) | 6.5(49.2) |
| Overall R(%)       | 32.3(4.2) | 51.2(4.8) | 34.0(2.6) |
| Fluo measurements  | 20.4 | 21.4 | 18.9 |
| Protein atoms      | 1911 | 1911 | 1911 |
| Ligand atoms (one molecule) | 8 (CYS) | 17(CYT) | 25 (CDP) |
| Carbohydrate atoms (NAG) | 14  | 14  | 14  |
| Water molecules    | 220 | 246 | 245 |
| R.m.s.d in bond lengths (Å) | 0.01| 0.01| 0.02 |
| R.m.s.d in bond angles (deg) | 1.08| 1.09| 2.0 |
| R.m.s.d in dihedral angles (deg) | 13.1| 12.4| 12.3 |
| Overall G factor   | 0.14| 0.15| –0.02|
| Wilson B-factor (Å²) | 24.1| 23.9| 21.8 |
| Mean B-factor for main chain atoms | 28.7 | 28.2 | 25.7 |
| Mean B-factor for side chain atoms and water oxygen atoms | 32.5 | 33.4 | 33.1 |
| Mean B-factor for all atoms | 30.8| 31.1| 29.7 |
| Residues in the most favored regions (%) | 93.2 | 92.7 | 93.2 |
| Residues in the additionally allowed regions (%) | 6.4 | 6.8 | 6.4 |
| Residues in the disallowed regions (%) (Leu 77 as part of type II β-turn) | 0.5 | 0.5 | 0.5 |

2.3. Binding studies using surface plasmon resonance spectroscopy

The surface plasmon resonance (SPR) measurements were carried out using Biacore-2000 (Pharmacia Biosensor AB, Uppsala, Sweden) at 25 °C in which a biosensor based system has been used for real time specific interaction analysis. The sensor chip CM5,
surfactant P20, the amine coupling kit containing N-hydroxysuccinimide (NHS), N-ethyl-N'-3(diethyl amino propyl) carbamide (EDC) and ethanolamine hydrochloride (Pharmacia Biosensor AB, Uppsala, Sweden) were used in the experiment. The immobilization of MbRIP1 was performed at a flow rate of 10 μl/min using amine coupling kit. The dextran on the chip was equilibrated with running buffer and carboxy methylated matrix was activated with EDC/NHS mixture and 200 μl of MbRIP1 (200 μg/ml) in 10 mM sodium acetate at pH 5.0, the unreacted groups were blocked by injecting ethanolamine (pH 8.5). The SPR signal for immobilized MbRIP1 reached 14357.8 RUs. Three concentrations of analytes, 1.0 μM, 2.0 μM and 4.0 μM were passed over immobilized MbRIP1 at a flow rate of 10 μl/min. The regeneration of protein surface from bound analyte was done using 10 mM glycine HCl at pH 3.0 (Fig. 2). The association ($K_{on}$) and dissociation ($K_{off}$) rate constants for the analytes bindings to MbRIP1 were calculated and the values of dissociation constants ($K_D$) were determined by mass action relation $K_D=K_{off}/K_{on}$ using BIAevaluation 3.0 software provided by the manufacturer.

2.4. Crystallization

The freshly purified and lyophilized samples of MbRIP1 were dissolved in 25 mM sodium phosphate buffer at pH 6.8 to a concentration of 20 mg/ml. CYS, CYT and CDP were also dissolved separately in the same buffer. The solutions of ligands were mixed separately with the protein solution in 1:1 (v/v) ratios and were incubated overnight at room temperature (~295 K). A solution containing 0.1 M sodium phosphate buffer pH 6.8 and 16% (v/v) polyethylene glycol 6000 (PEG-6000) was prepared as the reservoir buffer for the purpose of crystallization. It was mixed with each of the incubated solutions containing protein and ligand in 1:1 (v/v) ratio. The 6 μl drops of these solutions were prepared for the hanging drop vapor diffusion method at 298 K. After 10 days, cuboid shaped crystals from all the three sets of experiments were obtained.

2.5. X-ray intensity data collection and processing

Three X-ray intensity data sets were collected using one crystal each from the three samples containing complexes of MbRIP1 with (i) CYS, (ii) CYT, and (iii) CDP. The crystals were mounted in nylon loops and frozen in liquid nitrogen stream at 100 K. For data collection MAR CCD scanner (MAR Research, Norderstedt, Germany) was used on the BM14 Beam line at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). All the three data sets were indexed and processed using HKL-2000 package [16]. The summary of data collection statistics is given in Table 1.

2.6. Structure determination and refinement

The structures of the complexes of MbRIP1 with CYS (Fig. 3A), CYT (Fig. 4A) and CDP (Fig. 5A) were determined with molecular replacement method [17] using coordinates of native protein as the model (PDB: 3S9Q) [13]. It gave the clear solutions. All the three structures were refined with REFMAC 5 [18] from the CCP4i Version 4.2 program package [19,20] for 25 cycles. After these cycles of refinement, the $R_{cryst}$/ $R_{free}$ factors for the three complexes reduced to values of 0.251/0.282, 0.241/0.281 and 0.261/0.283, respectively. The models were improved by manual model
building with programs O [21] and Coot [22] on the graphics workstations. The difference (Fo/C0) Fourier maps calculated at this stage revealed extra densities at the substrate binding site in MbRIP1 into which molecules of CYS (Fig. 3B), CYT (Fig. 4B) and CDP (Fig. 5B) were fitted. These non-protein electron densities were observed at higher than 2.5σ. Out of these three compounds, the terminal phosphate of CDP is found in two conformations and is therefore modeled likewise as shown in Fig. 5B. The atoms of these compounds were included in the subsequent cycles of refinements. The same difference (Fo/C0) Fourier maps were also used for locating the positions of water oxygen atoms. The positions of water oxygen atoms were picked up based on the isolated electron densities at more than 2.5σ and having spherical shapes with distances up to 3.5 Å from nitrogen and oxygen atoms of protein. This gave 220, 246, and 245 water oxygen atom positions for the complexes with (i) CYS, (ii) CYT, and (iii) CDP respectively. After including all these atoms in the further cycles, the refinement converged to value of 0.179/0.204, 0.185/0.214 and 0.155/0.189 for Rcryst/Rfree factors for structures (i) to (iii) respectively. The final refinement statistics for all the three structures are included in Table 1. The refined atomic coordinates have been deposited in the Protein Data Bank (PDB) with accession codes of 4ZT8, 5CSO and 5CST, respectively.

3. Results

3.1. Analysis of binding studies using fluorescence spectroscopy

In order to determine the binding affinities of three ligands (i) CYS, (ii) CYT and (iii) CDP, the fluorescence properties of the binding site in MbRIP1 were exploited. The intrinsic fluorescence of MbRIP1 when excited at 280 nm is due to the presence of Tyr70 and Tyr111 in the substrate binding site. The fluorescence spectra were recorded in the presence of increasing amounts of ligands. The fluorescence intensities of MbRIP1 decreased gradually when the concentrations of ligands were increased, although the positions of emission maxima as well the shapes of the peaks remained unaltered. This suggested that the binding of ligands to MbRIP1 did not alter the environment in the vicinity of two aromatic residues. The observed fluorescence data were used for calculating the fluorescence quenching defined as Q% = (Fo−F)/Fo, where F is the fluorescence intensity measured after the ligands were added to the solution of MbRIP1 while Fo is the fluorescence intensity in the absence of ligands. Plots of graphs of Q(%) against the molar ratios of ligands to protein are shown in Fig. 1. The least-squares fits of the fluorescence intensity changes for the ligand/protein binding curves were obtained by Sigma Plot 8.0 [23]. The error bars on the experimental points were estimated from the average of values that were calculated using the binding equation described by Scatchard [24]. The approximate value of the dissociation constants (Kd) for the binding of (i) CYS, (ii) CYT and (iii) CDP were found to be 2.3 x 10−4 M, 5.0 x 10−6 M, and 2.8 x 10−7 M, respectively. These results indicated that all the three ligands bound to MbRIP1 with significant affinities.

3.2. Determination of binding affinity using SPR

The molecular interactions between MbRIP1 and its binding partners CYS, CYT and CDP, were studied in real time using SPR spectroscopy. The sensograms for the interactions with (i) CYS, (ii) CYT and (iii) CDP were shown in Fig. 2. The increase of resonance
units (RU) from the baseline represents the binding of ligands to the immobilized protein. The plateau line represents the steady state equilibrium phase of interactions between protein and the ligands whereas the decrease in RU from the plateau represents the dissociation phase. The values of dissociation constants \((K_D)\) were calculated to be \(4.1 \times 10^{-5} \text{ M}\), \(9.4 \times 10^{-7} \text{ M}\) and \(1.1 \times 10^{-7} \text{ M}\) for the binding of CYS, CYT and CDP respectively.

3.3. X-ray structural studies

3.3.1. Structure of the complex of MbRIP1 with cytosine

The structure of the complex of MbRIP1 with cytosine was determined at 1.98 Å resolution with 93.2% residues in the most favored regions of the Ramachandran map [25] as calculated using PROCHECK [26]. It may be mentioned here that the substrate binding site in the native structure contains ten water molecules which are interlinked through hydrogen bonds [16, PDB ID: 3S9Q]. Upon binding of cytosine, four out of ten water molecules were dislodged from the substrate binding site. The structure showed that cytosine formed five hydrogen bonds (Fig. 3C). The residues that form hydrogen bonds include Val69, Gly109 and Arg163 while Tyr111 interacted through a solvent water molecule. Cytosine also formed several van der Waals interactions. The notable van der Waals contacts were formed with residues, Tyr70, Tyr111, Asn110 and Gly109. The position of CYS in the substrate binding cleft is shown in Fig. 3D. It is clearly seen that cytosine is completely buried in the substrate binding pocket (Fig. 3E) with a buried surface area of 157 Å² as calculated using PISA server [21]. However, a significant space in the pocket was still unoccupied. The conformations as defined by torsion angles, \(\chi^1\) and \(\chi^2\) for the side chains of two important residues, Tyr70 and Tyr111 of the substrate binding site were estimated to be \(-70^\circ\) and \(158^\circ\), \(-81^\circ\) and \(140^\circ\), respectively.

3.3.2. Structure of the complex of MbRIP1 with cytidine

The structure of the complex of MbRIP1 with cytidine was determined at 1.78 Å resolution. As calculated using PROCHECK [26], it showed that the 92.7% residues were present in the most favored regions of the Ramachandran map [25]. Upon the binding of cytidine to MbRIP1, five out of ten water molecules were dislodged from the substrate binding site. The structure of the complex showed that the ligand is stabilized by nine hydrogen bonds (Fig. 4C). The residues that form hydrogen bonds include Ile71, Glu85, Tyr111, Glu160 and Arg163. Several van der Waals interactions have also been observed. The notable van der Waals contacts were formed with residues, Tyr70, Tyr111, Asn110 and Gly109. The orientation of cytidine in the substrate binding cleft can be seen in Fig. 4D. The cytidine molecule is fully buried in the cleft (Fig. 4E) with a buried surface area of 254.2 Å². In this case, the conformations of the side chains of Tyr70 and Tyr111 as defined by torsion angles, \(\chi^1\) and \(\chi^2\) were found to be \(-81^\circ\) and \(-172^\circ\), and \(-83^\circ\) and \(141^\circ\), respectively.

3.3.3. Structure of the complex of MbRIP1 with cytidine diphosphate

The structure of the complex of MbRIP1 with cytidine diphosphate (CDP) was determined at 1.78 Å resolution. As calculated using PROCHECK [26], the refined model has 93.2% of amino acid residues in the most favored regions of Ramachandran map [25]. Upon binding of CDP to MbRIP1, seven out of ten water molecules were dislodged. The map indicates that the beta phosphate has two conformations. The structure of the complex showed that CDP
formed at least 14 hydrogen bonds (Fig. 5C). It also formed several van der Waals contacts involving residues, Tyr70, Ile71, Tyr111, Asn110, Phe83, Gly109 and Glu112. The orientation of CDP in the substrate binding site can be seen from Fig. 5D. CDP is fitted well in the substrate binding cleft (Fig. 5E) with a buried surface area of 293.5 Å². In this case, the conformations of the side chains of Tyr70 and Tyr111 as defined by torsion angles, $\chi^1$ and $\chi^2$ were found to be $-77^\circ$ and $-162^\circ$, and $-77^\circ$ and $135^\circ$, respectively.

4. Discussion

The rectangular substrate binding cleft in type 1 RIPs is situated between two domains. The amino acid residues that constitute the substrate binding cleft include Tyr70, Ile71, Tyr111, Asn110, Phe83, Gly109 and Glu112. The orientation of CDP in the substrate binding site can be seen from Fig. 5D. CDP is fitted well in the substrate binding cleft (Fig. 5E) with a buried surface area of 293.5 Å². In this case, the conformations of the side chains of Tyr70 and Tyr111 as defined by torsion angles, $\chi^1$ and $\chi^2$ were found to be $-77^\circ$ and $-162^\circ$, and $-77^\circ$ and $135^\circ$, respectively.

Fig. 6. (A) Ten water molecules, W1 (526), W2 (525), W3 (524), W4 (272), W5 (520), W6 (453), W7 (263), W8 (427), W9 (390), W10 (273) were observed in the substrate binding cleft of the native unliganded structure of MbrriP1 (PDB ID: 3S9Q). The numbers in parentheses correspond to numbers in the PDB. (B) The binding of cytosine to MbrriP1 replaced four water molecules, W2, W3, W4 and W7. (C) The binding of cytidine replaced five water molecules, W1, W2, W4, W5 and W7. (D) Upon binding of cytidine diphosphate seven water molecules, W1, W2, W4, W5, W6, W7 and W10 were removed from the substrate binding site.

When cytidine diphosphate bound to MbrriP1, seven water molecules, W1, W2, W4, W5, W6, W7 and W10 were dislodged from the cleft (Fig. 6B) while the binding of cytosine removed five water molecules, W1, W2, W4, W5 and W7 (Fig. 6C). When cytidine diphosphate bound to MbrriP1, seven water molecules, W1, W2, W4, W5, W6, W7 and W10 were dislodged (Fig. 6D). This showed that three water molecules, W2, W4 and W7 were dislodged in all the structures indicating that it was a common subsite occupied by some parts of these compounds. The two water molecules, W8 and W9 remained present in all the structures indicating that these two locations were never occupied by these compounds. In the case of cytosine, two water molecules, W1 and W5 remained bound to protein while W3 was removed. In contrast, in both other structures, W1 and W5 were never observed while W3 was present. This indicated that the positioning of cytosine in the substrate binding cleft was slightly different from those occupied by other compounds. The molecule of cytidine diphosphate occupies the whole substrate binding site in MbrriP1 and thus suggests that the optimum size for fitting the compound in the substrate binding site corresponds to cytidine diphosphate.

The type 1 RIPs are known to remove an adenine residue from the sarcin/ricin loop (SRL) of rRNA in eukaryotic ribosomes [2,3]. The structural studies have also shown that whenever adenine containing nucleosides and nucleotides were used for crystallization of the complexes with MbrriP1, invariably, the complexes with adenine were obtained as these used to get hydrolyzed. It has also been shown that MbrriP1 binds to mRNA 5’cap structures with m$^7$GpppN where N is any base [12]. The present structures showed that MbrriP1 can also bind to cytosine and cytosine containing nucleoside and nucleotide. It is observed that cytosine, cytidine and CDP were fully buried in the cleft. It suggested that the optimum element for specificity in cytosine containing sequences of RNA structures is a nucleoside moiety. The number of interactions
observed with CDP were maximum indicating that it has the maximum binding affinity.

One of the main differences in the structure of protein upon binding of cytosine and cytosine containing compounds to MbrRIP1 as compared to those of adenine and guanine pertains to the conformation of the side chain of Tyr70. The values of the torsion angles, $\chi^1$ and $\chi^2$ for the side chain of Tyr70 in the complexes of cytidine and cytidine diphosphate were found centered at $-70^\circ$ and $-165^\circ$, respectively. The corresponding values in the complexes of MbrRIP1 with adenine and guanine were found to be centered at $-90^\circ$ and $-75^\circ$, respectively. The corresponding values in the unbound native structure were found to be $-66^\circ$ and $165^\circ$, respectively. Clearly, in the three structural states, the conformations of the side chain of Tyr70 are very different. It may be mentioned that the conformation of the side chain of Tyr111 remains the same in all the three states. The orientation of the plane of the aromatic ring of cytosine with respect to that of the aromatic ring of Tyr70 was $130^\circ$ while the corresponding angle in the complexes with adenine and guanine were centered at $165^\circ$. The plane of adenine ring in the cleft is oriented nearly parallel to the plane of the side chain of Tyr70 while that of cytosine is deviated considerably from parallel orientation. This difference is important for the catalytic activity of MbrRIP1. These differences may be responsible for the removal of adenine from the SRL of rRNA while cytosine cannot be removed because of different orientation in the cleft. However, it may act as an inhibitor of RIPs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.09.006.

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