Journal of the Taiwan Institute of Chemical Engineers 45 (2014) 764–771

Contents lists available at ScienceDirect

Journal of the Taiwan Institute of Chemical Engineers

journal homepage: www.elsevier.com/locate/jtice

Identifying the CmbT substrates specificity by using a quantitative structure–activity relationship (QSAR) study

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ARTICLE INFO

Article history:
Received 17 June 2013
Received in revised form 23 September 2013
Accepted 29 September 2013
Available online 4 November 2013

Keywords:
CmbT
Multidrug resistance
MFS general substrate transporter
Lactococcus lactis
QSAR

ABSTRACT

The CmbT substrate specificity and its role as a proton motive force-driven drug efflux pump at a molecular level were investigated in the study. In that order, 3D-quantitative structure–activity relationship (3D-QSAR) study was applied for selection of molecular determinants of multidrug recognition by CmbT.

CmbT multidrug resistance protein of *Lactococcus lactis* contributes to extruding the structurally, chemically, and pharmacologically diverse range of substrates out of bacterial cells. This function of CmbT may result in the failure of antibiotic therapy.

Homology model of CmbT protein was constructed and further optimized. The 3D-QSAR model predictive potential was proved by use of leave-one-out cross validation of the training set (Q^2: 0.69, $R^2_{\text{Observed vs. Predicted}}$: 0.918, RMSEE: 0.193) and verification set ($R^2_{\text{Observed vs. Predicted}}$: 0.704, RMSEP: 0.289).

The results obtained in this study showed that high CmbT affinities to ethidium, sulbactam, and sulfathiazole could be related to the absence of significant unfavourable interactions. In contrast, the presence of specific unfavourable interaction between two hydrogen bond donor groups in bacitracin, apramycin, novobiocin, vancomycin, kanamycin, gentamycin, and tobramycin is found to be the main reason for their lower CmbT affinities. In addition, membrane position of the CmbT binding site and positive correlation between substrates lipophilicity ($\log D_{\text{pH 5.0}}$) and CmbT affinity strongly indicates that CmbT recognizes its substrates within the membrane.

1. Introduction

Bacterial multidrug resistance (MDR) proteins play an important role in the resistance of bacterial cells to various cytotoxic structurally unrelated compounds such as heavy metals, organic solvents, dyes, disinfectants, and antibiotics [1–5]. The MDR transporters have been broadly classified into two major classes: the ATP-binding cassette (ABC) primary transporters, which use the hydrolysis of ATP as energy source for transport [6]; and the secondary transporters, driven by a proton or sodium motive force (pmf or smf) [7–11]. Studies on bacterial MDR transporters are becoming highly relevant during the last years since the MDR activities have been associated with the ongoing emergence of antibiotic resistance in pathogenic bacteria [12,13].

Although *Lactococcus lactis* is considered to be non-pathogenic and safe to use in starter cultures for cheese production, previous results showed the broad antibiotic specificity of LmrP, LmrA, LmrCD and the most recently described CmbT MDR transporters present in *L. lactis* [14–17]. MDR transporter could be associated with the mobile genetic elements implicating the possible transfer of the related genes to other bacteria present in food or the gastrointestinal tract, and as the final consequence, a serious threat to the efficacy of valuable antibiotics. Both the pmf-driven and the ABC transporters in *L. lactis* mediate resistance to toxic hydrophobic compounds, mainly cations and antibiotics, although several lactic acid bacteria also possess MDR transporters that mediate the extrusion of anionic antimicrobial compounds possibly conferring resistance to cholate [18,19].

While various experimental approaches have been used for prediction and characterization of the substrates for LmrP, LmrA

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1876-1070/$ – see front matter
http://dx.doi.org/10.1016/j.jtice.2013.09.033

and LmrCD [18,19], the computational technologies based on identification of structure–property relationships have been proven to be the most applicable for selection of molecular descriptors. For example, computations (*e.g.* docking) using the three-dimensional (3D) structure of the target and various quantitative structure–activity relationships (QSAR) models have been presented in the literature to predict the P-glycoprotein (P-gp) substrates [20–23].

The CmbT is a 454 amino acids integral membrane protein of *L. lactis* with 12–14 putative transmembrane spanning (TMS) domains that belongs to the major facilitator superfamily (MFS) [17]. The recent results revealed that the CmbT protein has an effect on host cell resistance to many structurally diverse compounds including lincomycin, cholate, sulbactam, ethidium bromide, Hoechst 33342, sulfadiazine, streptomycin, rifampicin, puromycin and sulfametoxazole [17]. Moreover, in vivo transport assays showed that CmbT-mediated extrusion of ethidium bromide and Hoechst 33342 is inhibited by the ionophores nigericin and valinomycin indicating the drug-proton antiport mechanism. However, the mechanism of multidrug transport by CmbT remains still unclear. In order to better understand the molecular basis of multidrug recognition by CmbT, in this study we intend to identify residues critical for multidrug recognition and to select molecular determinants of the CmbT substrate specificity.

2. Materials and methods

2.1. Experimental

2.1.1. Bacterial strains, plasmids, media

The following *L. lactis* strains were used in this study: NZ9000 [24], and two NZ9000 derivatives NZ9000/pNZ8113 and NZ9000/pCT50 carrying plasmids pNZ8113 and pCT50, respectively [17]. *L. lactis* strains were routinely grown in M17 broth (Merck, Germany) supplemented with 0.5% glucose (GM17) at 30 °C. When appropriate, the selectable media contained chloramphenicol (5 μg ml^{-1}). Agar plates were prepared by adding agar (1.5%, w/v) (Torlak, Serbia) to broth medium.

2.1.2. Growth studies and drug inhibition

The growth sensitivity of *L. lactis* NZ9000/pNZ8113 and *L. lactis* NZ9000/pCT50 to various toxic compounds was studied in the way described previously by Sakamoto et al. [25]. The experiment was performed essentially as given by Filipic et al. [17]. The culture was diluted again to an OD_{660} of 0.05 and aliquots (150 μl) were transferred into 96-well microtiter plates containing 150 μl of various concentrations of different toxic compounds (Table 1) in GM17 broth or GM17 broth supplemented with nisin (at a final concentration of 1 ng ml^{-1}) (Sigma, Germany). Due to the presence of a chloramphenicol resistance marker on plasmid pCT50, chloramphenicol was not included in the compound screening experiments. The growth rates of NZ9000/pNZ8113 and NZ9000/pCT50 (with or without nisin induction), as well as the concentrations of antimicrobial compounds, which inhibited the growth rate by 50% (IC_{50}), were determined using Graph Pad Prism 5 software. Data was fitted using non-linear regression analysis and least-square fitting of the absorbance data, where exponential growth equation and the general dose–response inhibition equation were applied. All experiments were done in triplicate.

2.2. Theoretical study

2.2.1. CmbT protein modelling, optimization and molecular dynamic study

The CmbT amino acid sequence (454 amino acids) was determined by http://supfam.org/SUPERFAMILY/cgi-bin/scop.cgi?sunidL'103473 [26]. Homology model of CmbT protein was obtained by use of the protein homology/analogy recognition engine V 2.0 (Phyre2) server [27]. A detailed description of the methods used by the Phyre server may be found in Bennett-Lovsey et al. [28]. The Phyre server uses libraries of known protein structures taken from the Structural Classification of Proteins (SCOP) database [29] and the protein data bank (PDB) [30]. The sequence of each of these structures is scanned against a nonredundant sequence database and a profile constructed and deposited in the 'fold library'. The known and predicted secondary structures of these proteins are stored in the fold library. The CmbT sequence was scanned against the nonredundant sequence database, and five iterations of PSI-Blast are used to gather both close and remote sequence homologs [28]. The accuracy of alignment between the CmbT protein sequence and a known template structure is key step in defining the accuracy of the final 3D model. Validated method for automating the assessment of alignment accuracy [31] has been implemented in Phyre2. Every position along the alignment where CmbT residue is matched to a template residue is assigned a score from the profile–profile matching algorithm [28]. Neighbouring high-scoring regions are indicative of accurate alignment and are colour-coded from red colour (very high confidence) to blue colour (very low confidence).

Fold recognition and alignment to proteins in the data banks were done (template used: d1pw4a, c2gfpA, c4apsB, c2xutc, c3o7pA, a1pv7a, c2g9pA, c3b9yA, c3v5uA, d3proc1, and c2vxhF), and a full three-dimensional model of the CmbT protein was constructed. The Schrödinger-Protein Preparation Wizard (Schrödinger, 2011 Protein Preparation Wizard, Epik version 2.2, Schrödinger, LLC, New York, NY, 2011, Impact version 5.7, Schrödinger, LLC, New York, NY, 2011, Prime version 3.0, Schrödinger, LLC, New York, NY, 2011), included in Maestro 2011 programme (Maestro, version 9.2, Schrödinger, LLC, New York, NY, 2011), was used for correction of possible structural problems, performing a restrained minimization, and creating reliable model of CmbT protein. The obtained model of CmbT protein was then further optimized by using the combination of OPLS force fields and GB/SA effective solvation model of the MacroModel programme (MacroModel, version 9.9, Schrödinger,

Table 1
Susceptibility of *L. lactis* NZ9000 and *L. lactis* NZ9000/pCT50 strains to various drugs.

Drug	IC_{50} of the indicated strain (μM)		Relative resistance[b]
	L. lactis NZ9000	*L. lactis* NZ9000/pCT50	
Ethidium bromide[a]	9.19 ± 3.18	21.80 ± 4.72	2.37
Cholate[a]	1316 ± 175	2827 ± 175	2.15
Hoechst 33342[a]	14.72 ± 4.11	24.52 ± 2.52	1.67
Kanamycin	9.56 ± 0.05	6.55 ± 1.16	0.68
Streptomycin[a]	41.22 ± 0.89	68.86 ± 2.96	1.67
Puromycin[a]	34.45 ± 1.04	41.64 ± 5.70	1.21
Rifampicin[a]	37.01 ± 16.28	55.20 ± 6.09	1.49
Lincomycin[a]	1.40 ± 0.07	2.01 ± 0.06	1.44
Vancomycin[a]	0.4470 ± 0.1252	0.3184 ± 0.1339	0.71
Erythromycin	0.05 ± 0.00	0.05 ± 0.01	1.04
Bacitracin	3.98 ± 0.18	4.14 ± 0.22	1.04
Cephalosporin C	0.054 ± 0.59	0.05 ± 0.64	0.96
Sulbactam[a]	40.02 ± 10.38	106.72 ± 8.89	2.67
Apramycin sulfate	26.12 ± 0.28	24.22 ± 0.24	0.93
Azithromycin	0.06 ± 0.10	0.05 ± 0.10	0.86
Novobiocin	0.16 ± 0.12	0.12 ± 0.13	0.74
Sulfamethoxazole[a]	83.22 ± 45.82	393.74 ± 118.36	2.34
Doxycycline	0.02 ± 0.10	0.02 ± 0.13	1.02
Sulfathiazole	7568.00 ± 0.16	13,298.00 ± 0.19	1.76
Tobramycin	17.78 ± 0.1779	5.962 ± 0.1727	0.34
Gentamycin sulfate	11.51 ± 0.1765	4.921 ± 0.1975	0.43

[a] Values taken from previous work [17].
[b] Relative resistance (RR) represents the increase-fold value of the *L. lactis* NZ9000/pCT50 resistance relative to control cells *L. lactis* NZ9000.