

Polycistronic Expression System for *Pichia pastoris* Composed of Chitinoand Chitosanolytic Enzymes

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Kaczmarek MB, Struszczyk-Swita K, Xiao M, Szczęsna-Antczak M, Antczak T, Gierszewska M, Steinbüchel A and Daroch M (2021) Polycistronic Expression System for Pichia pastoris Composed of Chitinoand Chitosanolytic Enzymes. Front. Bioeng. Biotechnol. 9:710922. doi: 10.3389/fbioe.2021.710922 Chitin is one of the most abundant biopolymers. Due to its recalcitrant nature and insolubility in accessible solvents, it is often considered waste and not a bioresource. The products of chitin modification such as chitosan and chitooligosaccharides are highly sought, but their preparation is a challenging process, typically performed with thermochemical methods that lack specificities and generate hazardous waste. Enzymatic treatment is a promising alternative to these methods, but the preparation of multiple biocatalysts is costly. In this manuscript, we biochemically characterised chitin deacetylases of *Mucor circinelloides* IBT-83 and utilised one of them for the construction of the first eukaryotic, polycistronic expression system employing self-processing 2A sequences. The three chitin-processing enzymes; chitin deacetylase of *M. circinelloides* IBT-83, chitinase from *Thermomyces lanuginosus*, and chitosanase from *Aspergillus fumigatus* were expressed under the control of the same promoter in methylotrophic yeast *Pichia pastoris* and characterised for their synergistic action towards their respective substrates.

Keywords: chitin, chitosan, enzymatic modification, polycistronic expression, self-processing 2A sequence, chitin deacetylase, chitinase, chitosanase

INTRODUCTION

Chitin is one of the most abundant, right after cellulose and lignin, naturally occurring biopolymer being the basic component of shells of crustaceans, insects and the cell walls of filamentous fungi (Al Sagheer et al., 2009; Marei et al., 2016; Ghormade et al., 2017). These are considered as burdensome waste products of marine and biotechnology industries. According to the report of the Food and Agriculture Organization of the United Nations (FAO), the estimated annual production of farmed shrimp only for Asian countries is approx. 2.5 million tons (FAO, 2017), of which 35–45% (w/w) are waste (shells and heads), which is about 1 million tons per year (Suryawanshi et al., 2019). Currently, over 60% of biotechnology companies use fungal organisms for various purposes, including brewing, wine and bakery industries, production of antibiotics and recombinant proteins for pharmaceutical applications, production of organic acids and recombinant enzyme proteins. It is estimated that the brewing industry in Brazil, the United States and China generates approximately 2.1 million tonnes of waste yeast biomass annually (Thiago et al., 2014). The China Pharmaceutical Industry Association reported that more than 80% of total antibiotic production is penicillin produced by the Ascomycota species *Penicillium* sp. This process produces 1.2 million tonnes of biowaste per year, mainly biomass of the above-mentioned moulds (Zhang et al., 2016). Wherein, the content of chitin in the cell wall of filamentous fungi may vary from 22 to 44% depending on the class of fungi (Synowiecki and Al-Khateeb, 1997; Chatterjee et al., 2005).

Despite interesting biological properties of chitin, its application is significantly limited primarily due to the highly crystalline structure of the polymer and the lack of solubility in commonly accessible solvents (Synowiecki and Al-Khateeb, 2003). Nowadays, products of chitin modification attract a significant interest. Their distinct physico-chemical properties, solubility in numerous media, increase the spectrum of their industrial applications. At present, N-deacetylated derivative of products of its chitin (chitosan) and degradation (chitooligosaccharides) are obtained by thermo-chemical and physical processes, which have several disadvantages (Hayes, 2012). The most important disadvantages are large amount waste that is harmful to the environment, the high energy consumption of processes and uncontrolled degradation of the polymer leading to the formation of a heterogeneous mixture of products with different physico-chemical properties, thereby affecting their biological properties (Kaczmarek et al., 2019).

Enzymatic modification of chitin and its derivatives may be a promising alternative to conventional methods. Biotransformation of chitin into chitosan through enzymatic deacetylation can be achieved with chitin deacetylases (EC 3.5.1.41). This enzymatic reaction has several advantages over the traditional chemical process, most importantly the production of chitosan with higher molecular weight and the desired degree of acetylation (Grifoll-Romero et al., 2018). Other enzymes involved in chitin and chitosan conversion are hydrolases: chitosanases (EC 3.2.1.132) and chitinases (EC 3.2.1.14). Both of them catalyse the hydrolysis of glycosidic bonds but differ in substrate specificity, the former hydrolysing bonds of chitosan and the latter of chitin (Nguyen et al., 2018). Due to the high specificities of these catalytic proteins, they are easy to control and result in products with a strictly defined chain distribution. Unfortunately, the multi-stage nature of these processes (deacetylation, depolymerisation), which are currently carried out in separated processes using disjointly operating biocatalysts, and the costs associated with obtaining each of them, make these methods economically uncompetitive.

Here we describe the synthesis of the first eukaryotic polycistronic expression system enabling the simultaneous expression of three genes encoding proteins with chitin- and chitosanolytic activity under the control of one promoter. The gene encoding chitin deacetylase from *Mucor circinelloides* IBT-83 strain was firstly heterologously expressed in yeast *Pichia pastoris* and biochemically characterised. Other genes encoding chitinase from *Thermomyces lanuginosus* (Zhang et al., 2015) and chitosanase from *Aspergillus fumigatus* (Chen et al., 2012) were synthesized and used for the preparation of a polycistronic expression vector. The functioning of the this multi-gene

system is based on the viral self-processing 2A sequences that have been identified in Foot and Mouth disease Viruses (FMDV) belonging to the *Picornaviridae* family (Donnelly et al., 2001b). These short peptide sequences (approx. 20 aa) inhibits the formation of a peptide bond between C-terminal glycine and proline, causing the phenomenon of so-called ribosomal skipping, and thus spontaneous initiation of the synthesis of a new polypeptide (Donnelly et al., 2001a). The presence of several sequentially acting biocatalysts in one reaction mixture is likely to result in cascade biocatalysis under *in vitro* conditions thanks to the decrease of the diffusion path of intermediate metabolic products between individual biocatalysts (Liu et al., 2014), and could result in the significant reduction of the costs of the process.

MATERIALS AND METHODS

Chemicals

The following reagents were purchased: fast digest restriction enzymes: *Not*I, *Sac*I and *Xba*I, Pierce BCA protein assay Kit, ATP solution (100 mM) (Thermo Scientific), Q5[®] High-Fidelity DNA polymerase, Thermostable *Taq* ligase, T4 polynucleotide kinase (New England Biolabs), Taq2x MasterMix (Tiangen), DNA Clean and ConcentratorTM-5 (Zymo Research), Wizard[®] SV Gel and PCR Clean-Up System and PureYieldTM Plasmid Miniprep System (Promega), Zeocin (Invitrogen), chitin from shrimp (*Pandalus borealis*), chitosan (MW 190–310 kDa) (Sigma Aldrich).

Strains Origins and Cultivation

The strain of filamentous fungi *Mucor circinelloides* IBT-83 from the culture collection of the Institute of Molecular and Industrial Biotechnology (Lodz, Poland) was used as a source of native chitin deacetylase *chdaI* and *chdaII* genes. Genes coding two native chitin deacetylases from *M. circinelloides* IBT-83 were isolated and molecularly cloned as described previously (Kaczmarek et al., 2016).

E. coli DH5 α transformants were grown in LB low salt medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl] supplemented with 25 µg/ml Zeocin. LB agar plates [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl 1.5% (w/v) agar], supplemented with 25 µg/ml of Zeocin, were used to screen the pPICZ α C bacterial transformant clones.

All culture media and ingredients used for *Pichia pastoris* experiments were prepared according to the protocol from the Easy select Pichia expression Kit (Invitrogen). Cells were grown in 250 ml baffled shake flasks covered with two layers of cotton gauze. *Pichia pastoris* KM71H strain was grown in YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose]. Transformed KM71H cells were grown in buffered minimal glycerol (BMD) medium [1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) Yeast Nitrogen base YNB, $4x10^{-5}\%$ (w/v) biotin and 1% (w/v) glycerol, 200 mM potassium phosphate buffer, pH 6.0] and buffered minimal methanol (BMM) medium [1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) Yeast, Nitrogen base YNB, $4x10^{-5}\%$ (w/v) biotin and 0.5% (w/v) methanol, 200 mM potassium phosphate buffer,

pH 6.0]. Transformed KM71H cells were selected on YPDS agar plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar] supplemented with 100 μ g/ml Zeocin.

Substrate Preparation

0.5% Chitosan in Hydrochloric Acid

The amount of 0.5 g of chitosan (MMW, DD 75–85%, viscosity 200 cP) was mixed with 95 ml of 0.1 mM HCl solution (pH 4.0) and left at room temperature overnight, with vigorous stirring. Next, concentrated HCl was added in small portions (50–100 μ l) to complete polymer dissolution, which was adjusted with 0.1 mM HCl to the final volume of 100 ml (pH 4.5).

2% Chitosan in Acetic Acid

The amount of 2 g of chitosan (MMW, 78.13 \pm 0.14%viscosity 200 cP) was mixed with 95 ml of 2% acetic acid and left at room temperature overnight, with vigorous stirring. Next, the solution was adjusted with 2% acetic acid to the final volume of 100 ml. The substrate prepared in this way was diluted to a concentration of 0.5% just before the reaction.

Colloidal Chitin

Colloidal chitin was prepared according to the previously published methodology (Souza et al., 2009). The amount of 5 g of chitin (chitin from shrimp *Pandalus borealis*, Sigma Aldrich) was mixed with 60 ml of concentrated HCl and left at room temperature overnight, with vigorous stirring. Next, 200 ml of ice-cold 95% ethanol was added to the mixture and incubated overnight at 4°C with vigorous stirring. The precipitate was collected by centrifugation at 5,000 g for 20 min at 4°C and transferred to a glass funnel with filter paper (80 g m⁻²). Colloidal chitin was washed with sterile distilled water until the substrate became neutral (pH 7.0). Colloidal chitin was freeze-dried and stored at 4 or -20°C (long term).

Multiple Sequence Alignment and Phylogenetic Analysis

The sequences of the catalytic domains (NodB) of the ChDaI and ChDaII proteins were included for multiple sequence alignment against other members of the CE4 deacetylase family. A multisequential comparison was made using Clustal Omega which use an alignment engine for aligning profile hidden Markov models (HMMs) to each other instead of the conventional dynamic programming and profile alignment (Sievers and Higgins, 2018). ChDaI and ChDaII were analysed with chitin deacetylase from Mucor rouxii MrCDA (Uniprot: P50325); chitin deacetylase from Rhizopus circinans RcCDA (Uniprot: A7UMZ0); chitin deacetylase from Gongronella butleri GbCDA (Uniprot: Q8J2N6); chitin deacetylase from Colletotrichum lindemuthianum ClCDA (PDB: 2IW0); chitin deacetylase from Pestolotiopsis sp. PesCDA (GenBank: APH81274.1); chitin deacetylase from Aspergillus nidulans AnCDA (PDB: 2Y8U); CE4 deacetylase isolated from the marine environment Arthrobacter sp. ArCE4A (PDB: 5LGC); peptidoglycan deacetylase from Streptococcus pneumoniae SpPgdA (PDB: 2C1G); CE4 esterase from Bacillus subtilis BsPdaA (PDB: 1W17); chitooligosaccharides deacetvlase from Vibrio

parahaemolyticus VpCOD (PDB: 3WX7); chitooligosaccharides deacetylase from Vibrio cholerae VcCOD (PDB: 4NY2); chitooligosaccharides deacetylase from Shewanella woodyi SbCOD (GenBank: ABN60929.1). Amino acid sequences of family CE4 CAZYmes (www.CAZY.org) were retrieved from Uniprot database, GeneBank and Protein Data Bank (PDB).

Chitin Deacetylase Activity Assay

Chitin deacetylase activity was determined based on the amount of acetic acid released during the chitosan deacetylation reaction. 1 ml of 0.5% (w/v) chitosan solution and 100 μ l of the supernatant obtained during proteins expression were preincubated for 2 min at 50°C, each in separate tube. The reaction was initiated by combining both the enzyme and the polymer solution and carried out for 120 min at 50°C in stirred (300 rpm) sterile 1.5 ml microcentrifuge tubes. Subsequently, the samples were incubated for 20 min at 80°C to inactivate the enzyme and cooled down. The amount of acetic acid released during the reaction was determined by GC-MS gas chromatography using Stabilwax[®]-DA; 30 m; 0.18 mm ID; 0.18 μ m column.

Effect of Temperature and Metal lons on Recombinant Chitin Deacetylase

To determine the optimum temperature for recombinant chitin deacetylase from *M. circinelloides* IBT-83, enzymatic reactions were performed at various temperatures ranging from 20 to 70°C. Reactions were performed according to the methodology described above. The effect of different metal ions on enzyme activity was verified by adding metal ions: Cu^{2+} , Fe^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} , Ni^{2+} to the enzymatic reaction to a final concentration of 1 mM or 10 mM.

Chitinase Activity Assay

Chitinase activity was determined as follows: 500 µl of 1% (w/v) colloidal chitin dissolved in citric buffer (pH 4.5) and 250 µl of the supernatant obtained during proteins expression were preincubated for 2 min at 50°C, each in separate tube. The reaction was initiated by combining both the enzyme and the polymer solution and carried out for 60 min at 50°C in stirred (550 rpm) sterile 1.5 ml microcentrifuge tube. Subsequently, the samples were incubated at 100°C for 5 min to inactivate the enzyme and cooled down. The amount of reducing sugars obtained during the reaction was determined using the Somogyi-Nelson method (Nelson, 1944).

Chitosanase Activity Assay

To chitosanase activity was determined according to the methodology described in section above. with the exception of the substrate, which in this case was 0.5% (w/v) chitosan in 2% (v/ v) acetic acid solution.

Determination of the Degree of Deacetylation

The degree of deacetylation of products obtained by the action of recombinant chitin deacetylases was determined using the method of potentiometric titration, according to the Broussignac method (Muzzarelli et al., 1997). Precipitated and lyophilised products of the reactions were dissolved in 0.02 M hydrochloric acid to a final concentration of 0.2% (w/v). The solutions were then titrated with 0.1 M NaOH. The two endpoints (PK) of titration observed in the diagram of $\Delta pH = f$ (V), corresponding to the titrant volumes V₁ and V₂ defined below, were determined by the first derivative method. For this purpose, plots of the dependence $\Delta pH/V = f$ (V) were prepared, where ΔpH and ΔV denote the increments between successive changes in pH and the volume of the titration reagent. The deacetylation degree was calculated from the following formula:

$$DD = \frac{M_{GlcNAc} \cdot c_{NaOH} \cdot (V_2 - V_1)}{m_{Ch} + c_{NaOH} \cdot (M_{GlcNAc} - M_{GlcN}) \cdot (V_2 - V_1)} \cdot 100\%$$
(1)

where: *DD*—degree of deacetylation (%), c_{NaOH} —titrant concentration (mol·dm⁻³), m_{Ch} —chitosan sample mass (g), V_1 —the volume of titrant used to neutralise hydrochloric acid (dm³), V_2 —the volume of titrant used to neutralise the sum of hydrochloric acid and protonated amine groups (dm³), M_{GlcNAc} —molar mass of: 2-acetamido-2-deoxy-D-glucopyranose (g·mol⁻¹), M_{GlcN} —molar mass of: 2-amino-2-deoxy-D-glucopyranose (g·mol⁻¹).

Chitin Deacetylases Expression Vectors Preparation

The chdaI and chdaII (without native signal peptide) genes used for heterologous expression in P. pastoris were amplified from previously described pJET transformants (Kaczmarek et al., 2016). The selected restriction sites: NotI and XbaI were incorporated into the PCR products by using specially designed primers (chdal_For 5'-ATTTGCGGCCGCTGACAC CTCCGCAAATTACTGG-3' chdaI_Rev 5'-TGCTCTAGAGC AAGTAACAAGGTAGCAATAAAGGCAG-3', chdaII_For 5'-ATTTGCGGCCGCTGCTACTTCCACCAAATCCGC-3', chdall_Rev 5'- TGCTCTAGAGCGAAAATGTAAGCAGCA ACGGC-3'). The resultant PCR products were cloned into the P. pastoris expression vector pPICZaC (Invitrogen) using available chemocompetent DH5a commercially cells (TianGen). The Zeocin resistant E. coli pPICZ chdaI and pPICZ_chdaII transformants were selected by culturing on LB agar plates, supplemented with 25 µg/ml of Zeocin at 37°C.

Polycistronic Vector Synthesis Generation of Plasmid for Polycistronic Expression

A polycistronic expression vector was prepared based on the commercial vector pPICZ α C. The *chda*II gene was amplified from previously described pJET transformants (Kaczmarek et al., 2016) using phosphorylated chdaII_FLCR 5'-GCTACT TCCACCAAATCCGCC-3' and chdaII_RLCR_5'-GAAAATGTAAGCAGCAACGGCA-3' primers. The gene coding chitinase from *Thermomyces lanuginosus* (Zhang et al., 2015) was enriched with the sequence coding the α -factor secretion signal, codon optimised and synthetised by Genewiz (Suzhou, China). The same approach was used for the gene

coding chitosanase from Aspergillus fumigatus (Chen et al., 2012). The sequences encoding the α -factor secretory sequences preceding both genes were optimised considering the degeneracy of the genetic code, limiting the occurrence of spontaneous homologous recombination. To generate 2A self-processing sequences, the ssDNA oligos were synthetised based on the P2A (ATNFSLLKQAGDVEENPGP) and T2A (EGRGSLLTCGDVEENPGP) amino acid sequences and hybridised according to the program 95°C-2min; 70°C-10 min; 72°C-5 min; 4°C-the end. The synthesis of the polycistronic vector was carried out using the LCR method (Kok et al., 2014). The above-described sequences used to synthesise the vector were amplified using phosphorylated primer sequences. Bridging oligonucleotides complementary to the ends of neighbouring DNA parts (half-bridging oligos) were designed with a target melting temperature (Tm). Figure 1A shows the map of the expression vector and a fragment of the expression cassette. The polycistronic vector pCHIT was synthesised from six DNA fragments, the size of which ranged from 50 bp to 3,500 bp. Individual fragments were amplified using the phosphorylated primers listed in the Supplementary Table S1.

Ligase Cycled Reaction-LCR

The LCR reaction was performed based on the modified method of Roth et al. Roth et al, (2014) as previously described by as (Liang et al., 2019). DNA fragments for a specific cycled ligation assembly reaction were mixed and diluted with ultra-pure H₂O to obtain a mixture with a concentration of 20 nM for each fragment. LCR assembly utilises single-stranded bridging oligos complementary to the ends of DNA parts to be assembled. The melting temperature of designed (half)bridging oligos was around 58-62°C. Bridging oligonucleotides (Supplementary Table S2) were diluted with ultra-pure H_2O to a final concentration of 200 µM. Thermostable Taq ligase (NEB) was used for the assembly reaction. The composition of the final reaction mixture (20 µL) is as follows: 2 nM for each DNA fragment, 10 nM bridging oligonucleotides, 1xTaq ligase buffer, 5% DMSO, and 40 U Taq ligase. The reaction was cycled according to the following program: initial denaturation 95°C (2 min) followed by 30 cycles of 95°C (30 s), 60°C (2 min), final incubation performed at 55°C (10 min), and samples stored at 4°C until transformed to E. coli DH5a (Tiangen, Beijing, China) according to the manufacturer's protocol. Figure 1B shows a simplified scheme for the synthesis of the polycistronic vector pCHIT using the LCR method.

Transformation of *Pichia pastoris* KM71H Strains and Selection of Positive Transformants

Plasmid DNA containing one of the genes of interest was linearised by overnight restriction digestion using *SacI* (Thermo Scientific). Purified, linearised plasmid (~100 ng) was



added into the electrocompetent KM71H cells. The prepared mixture was carefully transferred into the slide of the chilled and sterile cuvette. After electroporation (Voltage 1.5 kV, Resistance 125 ohms, Pulse length 3 msec.), cells were immediately resuspended in 500 μ l of 1 M cold sorbitol. Then 500 μ l of YPD pH 7.5 were added into the mixture. Cells were incubated with horizontal shaking at 30°C for at least 3–4 h. After transformation, cells were pelleted, 700 μ l of supernatant were removed, cells were resuspended in the remaining media and plated on YPDS agar plates supplemented with 100 μ g/ml Zeocine and incubated for 2 days at 30°C.

Colony PCR was used to confirm the presence of the expression cassette in the genome of *Pichia pastoris* transformants. Single colonies were replated on fresh YPD agar plates supplemented with 100 μ g/ml Zeocine and resuspended in 10 μ l sterilee water, heated to 95°C for 5 min, centrifuged at top speed and used as a template for PCR reaction containing Taq2x DNA MasterMix (Tiangen, Beijing, China), and universal primers (AOX1_For5'-GACTGGTTCCAATTGACAAGC-3', AOX1_Rev 5'-GCAAAT GGCATTCTGACATCC-3').

Pichia pastoris Cultivation Conditions

A single colony from fresh YPD agar plates supplemented with 100 μ g/ml Zeocine was used to inoculate 25 ml BMGY medium in a 250 ml baffled flask and grew at 28–30°C in a shaking incubator (250–300 rpm) for 18 h. The preculture with OD₆₀₀ ranging from 10 to 12 was used to inoculate 100 ml of BMGY medium in

1,000 ml baffled flask to final OD_{600} 0.2 and grew until the culture reaches $OD_{600} \sim 2-3$. The grown cells were harvested at 1.500–3.000×g for 5 min at room temperature and next resuspended in 20 ml of BMMY medium (100 ml baffled flasks covered with two layers of sterile gauze or cheesecloth). Every 24 h, pure methanol was added to the culture to the final concentration of 0.5%. The samples from 0, 24, 48, 72 and 96 h of culture were taken to measure protein concentration, prepare SDS-PAGE analysis and check the enzymatic activity of recombinant proteins.

RESULTS AND DISCUSSION

Chitin Deacetylases Multiple Sequence Alignment

Previous research has shown that the strain *Mucor circinelloides* IBT-83 is an efficient producer of intracellular chitin deacetylase (Kaczmarek et al., 2016). However, the large amount of microbial lipids produced by this strain significantly limited the efficiency of proteins purification and their enzymatic activity (Szczesna-Antczak et al., 2006). Therefore, two genes potentially encoding chitin deacetylases *chdaI* and *chdaII* were identified by bioinformatic analysis of the genome sequence of the filamentous fungi *Mucor circinelloides* CBS 277.49 (Project ID: 403122, Joint Genome Institute) using Blast-2.2.30 software. The *chdaI* and *chdaII* genes amplified on the cDNA from *Mucor*

							MTT			
							MIII			
		200		220		24	T(F/Y)DD	260		
		1						the second second		
ChDal	KA - PNISVRT	LDANGNLDTT	GYNTATDPDC	WWTATTCTSP	KISDINDDIS	KCPEPETWGL	TYDDGPN-CS	HNAFYDYLQE	QKLKASMFYI	190
Chdall	KA - PKIAVRK	LNSDGSINMT	GYDQNKDPDC	WWSASTCTKP	KLEGVNEDIY	TCPEPETWGL	TYDDGPN-CS	HNAFYDYLEA	EKIKASMFYI	204
MrCDA	KA - PNISVRT	LDANGNLDTT	GYNTATDPDC	WWTATTCTSP	KISDINDDIS	KCPEPETWGL	TYDDGPN-CS	HNAFYDYLQE	QKLKASMFYI	190
RCCDA	KA - PKIPVRK	AGSDGSLDMT	KYSDS - DPDC	WWSSSTCTKP	KHKDINEDIY	ACPEPETWGL	TYDDGPN-CS	HNAFYDYLEO	NKIKASMEYI	201
GhCDA	NGGPNISVRT	ENTDGSMNTN	GYDVANDPDC	WWTVSGCTVP	KLODVNADLY	KCPEPDTWGL	EYDDGPN-CS	HNAFYNELOF	ONLRASMEYI	179
CICDA			or brandr bo		VOVCTDII	OCTOPOLYAL	TYPOCOFTET	BOLLDILKO	NOVBATEEVM	76
CICDA					VPVGTPTL	QUTUPGEVAL	TTDDGPFIFI	-POLLDILKQ	NOVRATEEVN	15
PescDA					VPVGTIIT	ACTVPNTFAL	TEDDGPEAYT	- SELLDLLSS	NGVKATFFLN	72
AnCDA					VPTGQVIT	QCTTPNTIAL	TFDDGPSEYT	- PQLLDLLSR	YSARATFFVL	66
ArCE4A					V	DCATTKCVAL	TFDDGPGEYT	N-RLLDELSE	QHTPATFFVL	58
SpPgdA	FDYKDSQIIL	YPSPVVENLE	EIALPVSAFF	DVIQSSYLLE	KDAALYQSYF	DKKHQKVVAL	TFDDGPNPAT	TPQVLETLAK	YDIKATEEVL	270
BsPdaA					EKYDAEYL	GNTKEKTIYI	TEDNGYENGY	TPKVLDVLKK	HRVTGTEEVT	100
Vacon					ERTORITE	KGTIVI	TEDDORINAS	- I DV I NVI NO	EEVKATEVEN	26
vpcob						a TREATING	TFUUGFTNAS	- TOVINVLING	EEVICATETEN	30
Vecob						-SIPKGITYL	TEDDGPVNAS	-VEVIKVLNQ	GGVKATEYEN	40
SECOD					LCLASLSV	AHASDKTIYL	TFDDGPIDAT	- LGLLDALKD	ANIKATEFIN	58
				MT2						
				TIC TO-T						
	280		300	H(5/1)XXH	320		340		360	
ChDat	0011 111	DIND VOAN	DOWNDOWNIA	CHTWONDONT	TV		THOTH	FEVYTOKALK	LATOL TOD	0.40
ChDai	GSNVV	DWP YGAM	RGVVDGHHIA	SHIWSHPQMI	1		INQEVLA	EFTTIQKAIK	LAIGLIPR	249
Chdall	GSNVV	NWP YGAQ	RGQKAGHHIA	DHTWSHQLMT	TL		TNTEVLA	ELYYTQKAIK	MVTGVTPI :	263
MrCDA	GSNVV	DWP YGAM	RGVVDGHHIA	SHTWSHPQMT	TK		TNQEVLA	EFYYTQKAIK	LATGLTPR	249
RCCDA	GSNVV	NWP YGAQ	RGVKAGHHIA	DHTWSHOLMT	TL		TNDEVLA	ELYYTOKAIK	MVTGVTPL	260
GhCDA	GSNVM	MAR VGAM	ROVODCHHIA	FHTWSHOSIT	TI		TNOEGLA	FEVYTOKMIH	LATOVIDE	238
GUCDA	CHINE HILE IC	OND DTID	RUDADOIIIIA	CUTYAUDDLN			TROLOLA	CHIPOL SEATO	EATOVIER .	200
CICDA	GNINWANTEAG	SNP	RMRADGHLVG	SHITANPULN			SSAURIS	UMRULEEAIR	RIDGFAPK	139
PesCDA	GQNWGSIYDY	TS VVT	RMDAEGHQIG	SHTWSHADLA	TL		DAAGITS	QMTQLETALT	SILGKVPT	134
AnCDA	GDAAA	QNP GLLQ	RMRDEGHQVG	AHTYDHVSLP	SL		GYDGIAS	QMTRLEEVIR	PALGVAPA	125
ArCE4A	GKNVK	KYP KTLK	RMVDEGHQIG	SHTFDHKDIT	KL		TAEGIEH	EVQWTDEAIE	QAAGVKPQ	117
SpPadA	GKN	GNE DIVK	RIKSEGHVVG	NHSWSHPILS	01		SIDEAKK	OL TO TE OVI T	KVI GSSSK	329
BeDdaA	CH EVK	DOB	RHEDECHILC	NHEEHHDDIT	TV		TADOLOD	ELDEVNEEVV	KITCKODNI	160
DSFUAA	GHILLEFVK	DOPTTOLIK	RMSDEGHTTG	NHOFHHFULT	INTERPORT		TADQIQD	ELDSVINEEVI	KI IGRODIL-	100
VPCOD	AWHLDGIGDE	NEDRALEALK	LALDSGHIVA	NHSYDHMVHN	CVEEFGPNSA	AE - CNATGDH	QINSYQDPAY	DASMFAENLS	VLEKYLPNIT	125
VcCOD	AWHLDGIGDE	NEDRALEALK	LALDSGHIVG	NHSYDHMIHN	CVEEFGPTSG	AD - CNATGNH	QIHSYQDPVR	DAASFEQNLI	TLEKYLPTIR	129
SbCOD	AFHMYGEGDE	NEALAAVALQ	RLLDDGHILA	NHSYDHMLHN	CTD GVQSG	AVYCNQVGLW	PVKSYQDAAT	DFTYFVTNTA	KVVEIIPNAA	146
	LOOP	1			I	OOP 2				
	2001	MT2				0012			MTA	
		NIL S								
	R(I	P/x)(P/A)(Y/F/R)		400		420		Dx	xD(W/Y)	
Ch D-I	R(I	P/x)(P/A)(Y/F/R)		400 1		420 I		Dx	xD(W/Y)	
ChDal	R(1	Vx)(P/A)(Y/F/R)		400 1		420 I	VRWIASQLGL	Dx TAVIWNLD	xD(W/Y) TDDWSAGVTT	289
ChDal Chdall	R(I Y H	Vx)(P/A)(Y/F/R) WRPPYGD WRPAFGD		400 		420 I I DDR VDDR	VRWIASQLGL VRWIATQLGL	Dx TAVIWNLD TTILWDLD	TDDWSAGVTT	289 303
ChDal Chdall MrCDA	R(I	P/x)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPPYGD		400 		420 I VDR VDR IDDR	VRWIASQLGL VRWIATQLGL VRWIASQLGL	Dx TAVIWNLD TTILWDLD TAVIWNLD	TDDWSAGVTT TDDWSAGVTT TDDWAAGTSE TDDWSAGVTT	289 303 289
ChDal Chdall MrCDA RcCDA	R(I	P/x)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPPYGD WRPAFGD		400 		420 I I VDR VDR VDR	VRWIASQLGL VRWIATQLGL VRWIASQLGL VRWIATQLNL	Dx TAVIWNLD TTILWDLD TAVIWNLD TTVLWNLD	XD(W/Y) TDDWSAGVTT TDDWAAGTSE TDDWSAGVTT TDDWAAGSSK	289 303 289 300
ChDal Chdall MrCDA RcCDA GbCDA	R(I	P/x)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPAFGD WRAFGD WRAPYGD		400 		420 I I DDR VDDR IDDR VDDR VDDR	VRWIASQLGL VRWIATQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL	Dx TAVIWNLD TTILWDLD TAVIWNLD TTVLWNLD TTILWDLD	xD(W/Y) TDDWSAGVTT TDDWAAGTSE TDDWSAGVTT TDDWAAGSSK TNDWAAGSSK	289 303 289 300 278
ChDal Chdall MrCDA RcCDA GbCDA	R(I	P/x)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPAFGD WRAPYGD WRAPYGD		400 		420 I I DDR VDDR VDDR VDDR VDDR VDDR	VRWIASQLGL VRWIATQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL COGDIGGLGSL	Dx TAVIWNLD TTILWDLD TAVIWNLD TTVLWNLD TTILWDYD	xD(W/Y) TDDWSAGVTT TDDWSAGVTT TDDWSAGVTT TDDWAAGSSK TNDWQAGDGV TKDYGGDGV	289 303 289 300 278 176
ChDal Chdall MrCDA RcCDA GbCDA CICDA	R(I	Y(x)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPAFGD WRAFGD WRAPYGD MRAPYLS		400 I		420 I I DDR VDDR I DDR I DDR I DDR VDDR CDAG I DA VDDR	VRWIASQLGL VRWIATQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL CQGDLGGLGY	Dx TAVIWNLD TTILWDLD TAVIWNLD TTVLWNLD TTILWDYD HIIDTNLD	TDDWSAGVTT TDDWSAGVTT TDDWSAGTSE TDDWSAGVTT TDDWAAGSSK TNDWQAGDGV TKDYENN	289 303 289 300 278 176
ChDal Chdall MrCDA RcCDA GbCDA CICDA PesCDA	R(I	P(x)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPPYGD WRAPYGD MRAPYLS MRAPYLS		400		420 I I DDR V DDR V DDR V DDR V DDR V DDR C DAG V DDR	VRWIASQLGL VRWIATQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL CQGDLGGLGY ALSTLGGLGY	Dx TAVIWNLD TTILWDLD TAVIWNLD TTILWDYD TTILWDYD HIIDTNLD	TDDWSAGVTT TDDWSAGVTT TDDWSAGVTT TDDWSAGSK TDDWSAGDSK TNDWQAGDGV TKDYENN TLDYEHD	289 303 289 300 278 176 171
ChDal Chdall MrCDA RcCDA GbCDA CICDA PesCDA AnCDA	R(I	Vx)(P/A)(Y/F/R) WRPAFGD WRPAFGD WRPAFGD WRPAFGD MRAPYGD MRAPYLS MRPPYLE		400		420 I I VDDR VDDR VDDR VDDR CDAG CDAG I I NEL	VRWIASQLGL VRWIASQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL CQGDLGGLGY ALSTLGGLGY VLQVMRDLDY	Dx TAVIWNLD TTILWDLD TAVIWNLD TTVLWNLD TTILWDYD HIIDTNLD HVINANID RVISASVD	TDDWSAGVTT TDDWSAGVTT TDDWSAGVTT TDDWSAGVTT TDDWAAGSSK TNDWQAGDGV TKDYENN TLDYENQ	289 303 289 300 278 176 171 162
ChDal Chdall MrCDA RcCDA GbCDA CICDA PesCDA AnCDA ArCE4A	R(I	P/x)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPAFGD WRAPGD MRAPYGD MRAPYLS MRPPYFS MRPPYLS MRPPYLS MRPPYLS		400 		420 I DR VDDR VDDR VDDR VDDR VDDR CDAG CDAG I TNAL CAA GAH	VRWIASQLGL VRWIASQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL CQGDLGGLGY ALSTLGGLGY VLQVMRDLDY GAVYDRLIPY	Dx TAV IWNLD TTI LWDLD TAV IWNLD TTV LWNLD TTI LWDYD HI IDTNLD HV I NAN ID RV I SA SVD PLV LWDVD	TDDWSAGVTT TDDWSAGVTT TDDWSAGVTT TDDWSAGVTT TDDWAAGSSK TNDWQAGDGV TKDYENN TLDYENQ TKDYENQ TLDWKHH	289 303 289 300 278 176 171 162 151
ChDal Chdall MrCDA RcCDA GbCDA CICDA PesCDA ArCDA ArCE4A SpPgdA	R(1	%X)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPAFGD WRPAFGD WRAFGD WRAPYGD MRAPYGD MRAPYGD MRPPYFS MRPPYES LRPPY MRPPY		400 		420 I D R VDDR VDDR VDDR VDDR CDAG CDAG TNAL TNAL GAI	VRWIASQLGL VRWIASQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL CQGDLGGLGY ALSTLGGLGY VLQVMRDLDY GAVYDRLIPY TDDIRNSLDL	Dx TAV IWNLD TTI LWDLD TAV IWNLD TTU LWDLD TTI LWDYD HI IDTNLD HVI NANID RVI SA SVD PLV LWDVD SFI IMVD	TDDWSAGVTT TDDWSAGVTT TDDWSAGVTT TDDWSAGVTT TDDWAAGSSK TNDWQAGDGV TKDYEND TKDYEHD TKDYEHH SLDWKSK	289 303 289 300 278 176 171 162 151 363
ChDal Chdall MrCDA RcCDA GbCDA CICDA PesCDA ArCE4A SpPgdA BsPdaA	R(1	%X)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPAFGD WRPAFGD WRPAFGD WRPYGD WRPYS MRPPYS MRPPYLE LRPPY MRPPY MRPPY LRPPRGV		400 1		420 1 DR VDDR VDDR VDDR CDAG TNAL CAAG A CAAG C	VRWIASQLGL VRWIASQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL CGGDLGGLGY ALSTLGGLGY VLQVMRDLDY GAVYDRLIPY TDDIRNSLDL VLKETKRLGY	Dx TAV IWNLD TTI LWDLD TAV IWNLD TTV LWNLD TTI LWDYD TTI LWDYD RV I SASVD PLV LWDVD SF IMWDVD SF IMWDVD STV FWSVA	TDDWSAGVTT TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWAAGSSK TNDWAAGSSK TNDYENN TLDYENU TLDYEHH SLDWKSK	289 303 289 300 278 176 171 162 151 363 197
ChDai Chdail MrCDA RcCDA GbCDA CICDA PesCDA AnCDA AnCDA AnCCAA SpPgdA BsPdaA VoCCD	R(I	P/x)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPAFGD WRPAFGD WRPAFGD WRPYGD MRAPYGD MRAPYGS MRPPYFS MRPPYFS MRPPYFS MRPPYFS LRPPY LRPPY LRPPRV LRPPRV LRPPRV	VTKDFKADGL	400 	EPGYACDTAN	420 I DR VDDR VDDR VDDR VDDR VDDR CDAG CDAG I DR VDR CAA SAA PSNSVKAAIA	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL CQGDLGGLGY ALSTLGGLGY VLQVMRDLDY GAVYDRLIPY TDDIRNSLDL VLKETKRLGY VONILANNGY	Dx TAVIWNLD TTILWDLD TTVLWNLD TTILWDYD HIIDTNLD HIIDTNLD HVINANID RVISASVD SFIMWDVD SFIMWDVD GTVFWSVA GTHWDVDWAA	TDDWSAGYTT TDDWAAGTSE TDDWSAGYTT TDDWAAGSSK TNDWQAGDGV TKDYENN TLDYEHD TKDYEHD TKDYEHS SLDWKSK FVDWKISK FVDWKIN	289 303 289 300 278 176 171 162 151 363 197 215
ChDai Chdail MrCDA RcCDA CICDA ACCEA AnCDA ArCE4A SpFgdA BsPdaA VpCOD	R(1	%X)(P/A)(Y/F/R) WRPPYGD WRPPYGD WRPPYGD WRPPYGD WRAPYGD WRAPYGD WRAPYGD WRAPYGD WRPPYGV MRPPYLE LRPPY LRPPRGV ARLPYTNGWR	VTKDFKADGL	400 1	EPGYACDTAN EPGYACDTAN	420 1 DR VDDR VDDR VDDR CDAG CDAG CDAG CDAG A VDDR CA VDDR VDR V	VRWIASQLGL VRWIASQLGL VRWIASQLGL VRWIATQLNL CGGDLGGLGY ALSTLGGLGY VLOVMRDLDY GAVYDRLIPY TDDIRNSLDL VLKETKRLGY VONILANNGY	DX TAV IWNLD TTI LWDLD TAV IWNLD TTV LWNLD TTI LWDYD TTI LWDYD HVI INANID HVI NANID RVI SASVD PLV LWDVD SFI MWDVD SFI MWDVD GTHGWDVDWA	TDDWSAGVTT TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWAAGSSK TNDWAAGSSK TNDYENN TLDYENN TLDYENU TLDWKHH SLDWKSK FVDWKIN PENWGIAMPA	289 303 289 300 278 176 171 162 151 363 197 215 219
ChDai Chdall McCDA GbCDA ACCDA ACCDA ACCEA SpPgdA BsPdaA VpCOD VcCOD	R(1	%X)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPAFGD WRAFGD WRAFGD WRAPYGD MRAPYGS MRPPYFS MRPPYFS MRPPYFS MRPPYFS MRPPYFS MRPPYC RPPY RRPYT MRPPYC RRPYT MRPYT	VTKDFKADGL	400 T	EPGYACDTAN	420 1 DR 1 DR 1 DDR 1 DDR 1 DDR 0 DDR 0 DDR 0 DDR 0 CDAG 0 C	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL CQGDLGGLGY ALSTLGGLGY VLQVMRDLDY TDDIRNSLDL VLKETKRLGY VQNILANNGY VQNILANNGY	DX TAV IWNLD TTI LWDLD TAV IWNLD TTI LWDYD HI I DTNLD HI I DTNLD HV I NANID RV I SASVD SF I MWDVD SF I MWDVD SF I MWDVD GTH GWDVDWA GTH GWDVDWA GTH GWDVDWA	TDDWSAGVTT TDDWAAGTSE TDDWSAGVTT TDDWAAGSSK TNDWQAGDGV TKDYEND TKDYEND TKDYEND TKDYEND TKDYEKD TKDYEKD FVDWKISK FVDWKISK FVDWKISK	289 303 289 300 278 176 171 162 151 363 197 215 219
ChDal Chdall MrCDA RcDA GbCDA CICDA PesCDA ArCEA ArCEA SpPgdA VpCOD VcCOD SbCOD	R(1	%X)(P/A)(Y/F/R) WRPPAFGD WRPPAFGD WRPPAFGD WRPPYGD WRPPYGD WRPPYGD WRPPYES MRPPYLS MRPPYLS MRPPYLS MRPPYLS MRPPYLS MRPPYLS RRPPY LRPPRGV ARLPYTNGWR ARLPYTNGWR	VTKDFKADGL VTKDFGADGL VSSKLKGDGL	4000 	EPGYACDTAN EPGYVCDPAN DPAFACTAET	420 I I VDDR VDDR VDDR VDDR CDAG CDAG TNAL GAI STNSVKAAIA STNSSQVAVQ	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL CQGDLGGLGY VLQVMRDLDY GAVYDRLIPY TDDIRNSLDL VLKETKRLGY VQNILANNGY IQNMLAESDY	Dx TAVIWNLD TTILWDLD TVLWNLD TTULWDYD HIIDTNLD HVINAND RVISASVD PLVLWDVD SFIMWDVD QTVFWSVA QTHGWDVDWA KIFGWDFDWG	TDDWSAGVTT TDDWSAGVTT TDDWSAGVTT TDDWSAGTSE TDDWSAGSSK TNDWQAGDGV TKDYENU TLDYENU TLDYENU TLDYENU TLDYENU FVDWKIN PENWGIAMPA PENWGIAMPA	289 303 289 300 278 176 171 162 151 363 197 215 219 236
ChDai Chdail McCDA GbCDA CICDA PesCDA ArCEA SpPgdA BsPdaA VpCCD VcCOD SbCOD	R(1	P/x)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPPYGD WRPPYGD WRPPYGD WRPPYGS MRPPYLS MRPPYLS MRPPYLS MRPPYCS MRPPYLS MRPPYLS MRPPYLS MRPPYLS MRPPYLS MRPPYLS MRPPYLS	VTKDFKADGL VTKHFQADGL VSSKLKGDGL	400 1 CATSDDLKPW CATSDDLKPW CATSDNLKPW CATSDNLKPW LOOP 3	EPGYACDTAN EPGYVCDPAN DPAFACTAET	420 1 DR 1 DR 1 DDR 1 DDR 1 DDR 1 DDR 1 DDR 1 DR 1 DR	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL VRWIATQLNL QGDLGGLGY ALSTLGGLGY VLQVMRDLDY TDDIRNSLDL VLKETKRLGY VQNILANQGY IQNMLAESDY	DX TAV IWNLD TTI LWDLD TAV IWNLD TTI LWDLD TTI LWDLD TTI LWDLD HI ID TNLD HI ID TNLD HI ID TNLD HI ID TNLD SF I MWDVD SF I MWDVD SF I MWDVD GTH GWD VDWA GTH GWD VDWA KI F GWD FDWA KI F GWD FDWA	xD(W/Y) TDDWSAGVTT TDDWSAGTTT TDDWAAGTSE TDDWAAGSSK TNDWAAGDGV TKDYE NN TLDYE NN TLDWK SK FVDWK SK FVDWK SK PENWG I AMPA PENWG I AMPA PEDWGVAFPA LOOP4	289 303 289 300 278 176 151 162 151 363 197 215 219 236
ChDal Chdall MrCDA RcDA GbCDA CICDA PesCDA ArCEA ArCEA SpPgdA VpCOD VcCOD SbCOD	R(1	%X)(P/A)(Y/F/R) WRPPYGD WRPPYGD WRPPYGD WRPPYGD WRPYGD WRPYGD WRPYGD WRPYGD WRPYGE MRPPYLS MRPPYLS MRPPYLS LRPPRY LRPPRGV ARLPYTNGWR ARLPYTNGWR	VTKDFKADGL VTKHFQADGL VSSKLKGDGL	4000 	EPGYACDTAN EPGYVCDPAN DPAFACTAET	420 I I UDR VDDR VDDR VDDR VDDR CDAG CDAG I CDAG CDAG CDAG CDAG CDAG STNSCASIQ STNSSQVAVQ I5	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL QGDLGGLGGLGY VLQVMRDLDY GAVYDRLIPY TDDIRNSDL VLKETKRLGY VQNILANNGY VQNILANNGY	Dx TAVIWNLD TTILWDLD TAVIWNLD TTULWDYD HIIDTNLD HVINANID RVISASVD PLVLWDVD SFIMUDVD GTHGWDVDWA GTHGWDVDWA KIFGWDFDWG	XD(W/Y) TDDWSAGVTT TDDWSAGVTT TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWQAGDGV TKDYE NN TLDYE HD TKDYE SK FVDWK SK FVDWK I SK FVDWK I SK PENWGI AMPA PEDWGVAFPA LOOP 4	289 303 289 300 278 176 171 162 151 363 197 215 219 236
ChDal Chdall McCDA RcCDA GbCDA ACCDA ACCDA ACCE4A SpPgdA BsPdaA BsPdaA SpCOD VcCOD SbCOD	R(1	P/x)(P/A)(Y/F/R) WRPPAFGD WRPPAFGD WRPPAFGD WRPAFGD WRPPYGD WRPPYS MRPPYS MRPPYE LRPPY MRPPY RRPPY RRPPY RRPPY RRPPY RRPPY RRPPY RRPYTNGWR ARLPYTNGWR ARLPYTNGWR	VTKDFKADGL VTKHFQADGL VSSKLKGDGL	400 1 CATSDDLKPW CATSDDLKPW CATSDNLKPW LOOP3	EPGYACDTAN EPGYACDTAN DPAFACTAET Ta	420 1 DR 1 DR 1 DDR 1 DDR 1 DDR 1 DDR 1 DDR 1 DDR 1 DDR 1 DDR 1 DDR 1 DR 1 DR	VRWIASQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL VRWIATQLNL QGDLGGLGY ALSTLGGLGY VLQVMRDLDY TDDIRNSLDL VLKETKRLGY VQNILANNGY VQNILANNGY IQNMLAESDY	DX TAV IWNLD TTI LWDLD TAV IWNLD TTU LWDYD TTI LWDYD HI D TNLD HV INANID RVI SASVD SFI MWDVD SFI MWDVD GTVFWSVA GTHGWDVDWA GTHGWDVDWA KI FGWD FDWG	XD(W/X) TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWAAGSSK TNDWAGGCV TKDYE NN TLDYE NN TLDYE NN TLDYE NH SLDWK SK FVDWK I NH SLDWK SK FVDWK I NH SLDWK SK FVDWK I NK FVDWK I NK FVDWK I NK TLDYE -	289 303 289 300 278 176 171 162 151 363 197 215 219 236
ChDai Chdail MrCDA GbCDA CICDA PesCDA AnCDA AnCDA AnCDA AnCDA SpPdA SpPdA VpCOD VcCOD SbCOD	R(1	P/x)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPAFGD WRPAFGD WRPAFGD WRPYGD MRAPYLS MRPPYFS MRPPYFS MRPPYLS RPPYLS RPPYLS RRPPYLS RRPPYLS RRPPYLS RRPPYLS RRPYTROWR ARLPYTNGWR ARLPYTNGWR	VTKDFKADGL VTKHFQADGL VSSKLKGDGL	4000 T CATSDDLKPW CATSDNLKPW CATSDNLKPW LOOP 3	EPGYACDTAN EPGYVCDPAN DPAFACTAET M La	420 I I I VDDR VDDR VDDR VDDR VDDR CAAS CAAS I STNSU VDR A VDDR VDR VDR VDR VDR VDR VDR VD	VRWIASQLGL VRWIASQLGL VRWIASQLGL VRWIATQLNL QGDLGGLGY ALSTLGGLGY VLQVMRDLDY GAVYDRLIPY TDDIRNSLDL VLKETKRLGY VQNILANQGY IQNMLAESDY	Dx TAVIWNLD TTILWDLD TAVIWNLD TTILWDYD HIIDTNLD HIIDTNLD HVINANID RVISASVD SFIMWDVD SFIMWDVD GTVFWSVA GTHGWDVDWA GTHGWDVDWA KIFGWDFDWG	XD(W/Y) TDDWSAGYTT TDDWAAGTSE TDDWSAGVTT TDDWAAGSSK TNDWQAGDGVT TKDYENN TLDYEND TKDYEND TLDYEHD TKDYENK FVDWKISK FVDWKISK FVDWKISK PENWGIAMPA PENWGIPMPA PEDWGVAFPA LOOP4	289 303 289 300 278 176 171 162 151 363 197 215 219 236
ChDal Chdall MrCDA RcCDA GbCDA CICDA PesCDA ArCE4A BsPdaA VpCOD VcCOD SbCOD	R(1	DY1)(P/A)(Y/F/R) WRPP4FGD WRPP4FGD WRPAFGD WRP4FGD WRAPYGS MRPPYES MRPPYES MRPPYES ARLPYTNGWR ARLPYTNGWR ARLPYTNGWR	VTKDFKADGL VTKHFQADGL VSSKLKGDGL FANSGN-	400 1 CATSDDLKPW CATSDDLKPW CATSDNLKPW LOOP 3	EPGYACDTAN EPGYVCDPAN DPAFACTAET La	420 1 DDR 	VRWIASQLGL VRWIASQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL VRWIATQLNL QGDLGGLGY ALSTLGGLGY VLQVMRDLDY TDDIRNSLDL VLQVMRLIPY TDDIRNSLDL VLKETKRLGY VQNILANNGY VQNILANNGY VQNILANNGY SQN IQNMLAESDY -SLAVENLPK	Dx TAV IWNLD TTI LWDLD TAV IWNLD TTV LWNLD TTI LWDYD TTI LWDYD RV I SASVD PLV LWDVD SF I MWDVD GTV FWSVA GTHGWDVDWA GTHGWDVDWA KI FGWD FDWG	XD(W/X) TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWAAGSSK TNDWAAGSSK TNDYAAGSSK TNDYE NN TLDYE NN TLDYE NN TLDWK HH SLDWK SK PENWG I AMPA PENWG I AMPA LOOP 4 SK0 VATCYN I SHP	289 303 289 300 278 176 171 162 151 363 197 215 219 236
ChDail Chdail MrCDA GbCDA CICDA PesCDA AnCDA AnCDA AnCDA AnCDA SpPgdA BsPdaA VpCOD VcCOD SbCOD	R(I	P/x)(P/A)(Y/F/R) WRPPYGD WRPAFGD WRPPYGD WRAPYGD WRAPYGD WRAPYGS MRPPYES MRPPYES MRPPYES MRPPYES MRPPYES ARLPYTNGWR ARLPYTNGWR ARLPYTNGWR ARLPYTNGWR DYIAMGTNGT	VTKDFKADGL VTKHFQADGL VSSKLKGDGL FANSGN	400 T	EPGYACDTAN EPGYVCDPAN DPAFACTAET M Ls 	420 1 DT 1	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL CQGDLGGLGY ALSTLGGLGY VLQVMRDLDY GAVYDRLIPY TDDIRNSLDL VLKETKRLGY VQNILANQGY IQNMLAESDY -SLAVENLPK TIAMEELPK	Dx TAVIWNLD TTILWDLD TAVIWNLD TTILWDYD HIIDTNLD HVINANID RVISASVD GTVFWSVA GTHWDVD SFIMWDVD SFIMWDVD GTHGWDVDWA GTHGWDVDWA KIFGWDFDWG	XD(W/X) TDDWAAGTSE TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWQAGDGVT TKDYENN TKDYENN TKDYEND TKDYENSK FVDWKI-	289 303 289 300 278 176 171 162 151 363 197 215 219 236 356 356
ChDal Chdall MrCDA RcDA GbCDA ClCDA PesCDA ArCE4A BsPdaA VpCOD VcCOD SbCOD	R(1	DY1(P/A)(Y/F/R) WRPP4FGD WRP4FGD WRP4FGD WRP4FGD WRAPYGD MRPPYES MRPPYES MRPPYES ARLPYTNGWR ARLPYTNGWR ARLPYTNGWR DYIAMGTNGT DFIEMGSNGT	VTKDFKADGL VTKHFQADGL VSSKLKGDGL FANSGN FNNSGQ	400 1 CATSDDLKPW CATSDDLKPW CATSDNLKPW LOOP 3	EPGYACDTAN EPGYVCDPAN DPAFACTAET La IVLT	420 1 DDR 	VRWIASQLGL VRWIASQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL VRWIATQLNL QGDLGGLGY ALSTLGGLGY VLQVMRDLDY TDDIRNSLDL VLQVMRLIPY TDDIRNSLDL VLKETKRLGY VQNILANNGY VQNILANNGY VQNILANNGY VQNILANNGY VQNILANNGY VQNILANNGY VQNILANGY VQNI VQNI VQNI VQNI VQNI VQNI VQNI VQNI	Dx TAV IWNLD TTI LWDLD TAV IWNLD TTV LWNLD TTV LWNLD TTI LWDYD RV I SASVD PLV LWDVD SF I MWDVD SF I MWDVD GTV FWSVA GTHGWDVDWA GTHGWDVDWA ATHGWDVDWA KI F GWD FDWG	XD(W/X) TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWAAGSSK TNDWAAGSSK TNDYANN TLDYEND TKDYEND TLDWKSK FVDWKIN PENWGIAMPA PENWGIAMPA LOOP4 Sto VATCYNISHP VATCMNITNP	289 303 289 300 278 176 171 162 151 363 197 215 219 236 356 370
ChDai Chdail McCDA GbCDA ACCDA ACCEA AFCEA SpPgdA BsPdaA VQCOD VcCOD SbCOD ChDai ChDai	R(1	DY1AMGTNGT DY1AMGTNGT	VTKDFKADGL VTKHFQADGL VSSKLKGDGL FANSGN	400 T	EPGYACDTAN EPGYVCDPAN DPAFACTAET M Ls 	420 I DDR VDDR VDDR VDDR CDAG CDAG CDAG CDAG CDAG CDAG STNSQVAQ STNSQVAQ STNSSQVAQ STNSSQVAVQ HEI - NNTTM HEI - NTTM	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL CQGDLGGLGY ALSTLGGLGY VLQVMRDLDY TDDIRNSLDL VLKETKRLGY VQNILANQGY IQNMLAESDY -SLAVENLPK -SLAVENLPK	Dx TAVIWNLD TTILWDLD TAVIWNLD TTILWDYD HIIDTNLD HVINANID RVISASVD SFIMWDVD SFIMWDVD SFIMWDVD GTHGWDVDWA GTHGWDVDWA KIFGWDFDWG	XD(W/X) TDDWAAGTSE TDDWAAGTSE TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWAAGSGVTT TKDYE NN TKDYE NN TKDYE NN TKDYE NN TLDYE SK FVDWK SK FVDWF SK FVD	289 303 289 278 176 171 162 151 363 197 215 219 236 356 370 355
ChDal Chdall MrCDA RcDDA GbCDA ClCDA PesCDA ArCE4A SpPgdA VpCOD VcCOD SbCOD ChDal ChDal Chdall MrCDA RcCDA	R(1	DY1AMGTNGT DY1AMGTNGT DY1AMGTNGT DY1AMGTNGT	VTKDFKADGL VTKHFQADGL VSSKLKGDGL FANSGN FANSGQ FATSGQ	400 1 CATSDLKPW CATSDNLKPW CATSDNVPPW LOOP3	EPGYACDTAN EPGYVCDPAN DPAFACTAET IX IVLT IVLT IVLT	420 1 DDR 	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL VRWIATQLNL VRWIATQLNL QGDLGGLGY ALSTLGGLGY VLQVMRDLDY TDDIRNSLDL VLQVMRLIPY TDDIRNSLDL VLKETKRLGY VQNILANNGY IQNMLAESDY IQNMLAESDY -SLAVENLPK -SLAWEYLPK	Dx TAV IWNLD TTI LWDLD TAV IWNLD TTV LWNLD TTV LWNLD TTI LWDYD RV I SASVD PLV LWDVD SF I MWDVD SF I MWDVD GTVFWSVA GTHGWDVDWA KIFGWDFDWG HISAYKQV I D I SAAYKNVVD I KAAYKNVVD	XD(W/X) TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWAAGSSK TNDWAAGSSK TNDYENN TLDYENN TLDYENH SLDWKSK FVDWKINH SLDWKSK PENWGIAMPA PENWGIAMPA LOOP4 S40 VATCYNISHP VATCYNISHP VATCYNISHP	289 303 289 300 278 176 171 162 151 363 197 215 219 236 356 370 355 355 355
ChDai Chdail McCDA RcCDA GbCDA CICDA PesCDA ArCE4A SpPgdA BsPdaA SpPgdA BsPdaA VQCOD VcCOD SbCOD ChDai ChDai ChDai ChDai ChDai ChDai	R(I	DY1AMGTNGT DY1AMGTNGT DY1AMGTNGT SYVEMGSNGT	VTKDFKADGL VTKHFQADGL VSSKLKGDGL FANSGN FANSGQ FANSGQ FATSGQ FATSGQ	400 T CATSDDLKPW CATSDNLKPW CATSDNLKPW LOOP 3	EPGYACDTAN EPGYVCDPAN DPAFACTAET M Lx 	420 1 DT 1	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL CaGDLGGLGY ALSTLGGLGY VLQVMRDLDY TDDIRNSLDL VLKETKRLGY VQNILANQGY IQNMLAESDY -SLAVENLPK -SLAVENLPK -SLAVENLPK -SLAMEYLPK -SLAMEYLPK	Dx TAV IWNLD TTI LWDLD TAV IWNLD TTI LWDLD TTI LWDLD HI ID TNLD HI ID TNLD HV INANID SF I MWDVD SF I MWDVD SF I MWDVD GTHGWDVDWA GTHGWDVDWA KI FGWD FDWG I ISAYKQV ID I RAAYKNVLD I SAYKQV ID I KAAYKNVVD	XD(W/X) TDDWSAGVTT TDDWSAGVTT TDDWSAGVTT TDDWSAGSVTT TDDWAAGSSK TNDWQ-AGDGV TKDYEND TLDYEND TLDYE	289 303 289 300 278 176 151 162 151 215 219 236 356 370 355 367 345
ChDai Chdail McCDA RcCDA GbCDA ClCDA PesCDA ArCE4A SpPgdA VpCOD VcCOD SbCOD ChDai ChDai McCDA RcCDA GbCDA ClCDA	R(1	DY1AMGTNGT WRPAFGD WRPAFGD WRPAFGD WRAPYGD WRAPYGD WRAPYGD WRAPYGD WRAPYGD WRAPYGD KRPPY LRPPY LRPPY ARLPYTNGWR ARLPYTNGWR ARLPYTNGWR ARLPYTNGWR DYIAMGTNGT DYIAMGTNGT DYIAMGTNGT SYVEMGSNGT EFIQMGNNGS EFIQMGNNGS	480 VTKDFKADGL VTKHFQADGL VSSKLKGDGL FANSGN FANSGQ FATSGQ MASGGN VGANSY	400 1 CATSDDLKPW CATSDNLKPW CATSDNLPW LOOP 3	EPGYACDTAN EPGYVCDPAN DPAFACTAET IX 	420 	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL VRWIATQLNL VRWIATQLNL VRUIATQLNL VLGYMRDLDY GAUYDRLIPY TDDIRNSLDL VLGYKRLGY VQNILANNGY IQNMLAESDY IQNMLAESDY -SLAVENLPK -SLAVENLPK -SLAWEYLPK -SLAWEYLPK -QLAVENIPN	DX TAV IWNLD TTI LWDLD TTV LWNLD TTV LWNLD TTV LWNLD TTV LWNLD TTV LWNLD RVI SAS VD PLV LWDVD SF IMUDVD SF IMUDVD GTV FWSVA GTHGWDVDWA KIFGWDFDWG HISAYKQVID ISAAYKNVLD ISAAYKNVLD ISAAYKNVLD ISAAYKNVLD ISAAYKNVLD ISAAYKNVLD ISAAYKNVLD ISAAYKNVLD ISAAYKNVLD	XD(W/X) TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWAAGSSK TNDWAAGSSK TNDYENN TLDYENN TLDYENH SLDWKSK FVDWKIN PENWGIAMPA PENWGIAMPA LOOP4 S40 VATCYNISHP VATCMNITYP VATCMNITYP VATCMNITYP	289 303 289 300 278 176 171 162 151 363 197 219 236 356 370 355 367 345 243
ChDal Chdall MrCDA RcCDA GbCDA ArCEA SpPgdA BsPdA BsPdA BsPdA BsPdA BsPdA BsPdA BsPdA RsCDA ChDal Chdall MrCDA RcCDA GbCDA ChDal	R(I	DY1AMGTNGT DY1AMGTNGT DY1AMGTNGT SYVEMGSNGT SYVEMGSNGT SYVEMGSNGT SYVEMGSNGT SYVEMGSNGT SYVEMGSNGT SYFNELSAD	480 VTKDFKADGL VTKHFQADGL VSSKLKGDGL FANSGN FANSGQ FATSGQ FATSGQ MASGGN GGT	400 1 CATSDDLKPW CATSDNLKPW CATSDNLKPW LOOP 3	EPGYACDTAN EPGYVCDPAN DPAFACTAET IVLT 	420 	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL VRWIATQLNL VRWIATQLNL VRWIATQLNL VRWIATQLNL VLWIATQLNL VLWIATQLNU VLWIASQLGY VQNILANQGY IQNMLAESDY VQNILANQGY IQNMLAESDY S200 -SLAVENLPK -SLAVENLPK -SLAWENLPK -SLAWENLPK -SLAWENLPK -SLAWENLPK VSLTQKLIDT	DX TAV IWNLD TTI LWDLD TAV IWNLD TTI LWDLD TTI LWDLD TTI LWDLD HI D TNLD HV INANID RV ISASVD SFI MVDVD SFI MVDVD SFI MVDVD GTHGWDVDWA GTHGWDVDWA GTHGWDVDWA KIFGWD FDWG ISAYKQVID ISAAYKNVLD ISAYKQVID ISAYCOV	XD(W/X) TDDWAAGTSE TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWAAGSGVTT TDDWAAGSSK TNDYE NN TLDYE NN T	289 303 289 300 278 176 171 162 151 363 197 215 219 236 356 370 356 370 355 367 345 243
ChDai ChDai MCDA RcDA GbCDA CICDA PesCDA ArCEA ArCEA SpPgdA VpCOD VcCOD SbCOD ChDai ChDai ChDai ChDai ChDai ChDai ChDai	R(1 	DY1AMGTNGT WRPAFGD WRPAFGD WRPAFGD WRAPYGD WRAPYGD WRAPYGD WRAPYGS MRPPYLS LRPPY LRPPY LRPPY ARLPYTNGWR ARLPYTNGWR ARLPYTNGWR ARLPYTNGWR ARLPYTNGWR TFIEMGSNGT DY1AMGTNGT SYVEMGSNGT EFIOMGNNGS EKFNNELSAD TNFQNGLAS	480 VTKDFKADGL VTKHFQADGL VSSKLKGDGL FANSGN FANSGN FATSGQ MASGGN GGT	400 1 CATSDDLKPW CATSDNLKPW CATSDNVPPW LOOP3	EPGYACDTAN EPGYVCDPAN DPAFACTAET IVLT 	420 	VRWIASQLGL VRWIASQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL VRWIATQLNL VRWIATQLNL VRWIATQLNY CAGDLGGLGY ALSTLGGLGY VLOVMRDLDY GAVYDRLIPY TDDIRNSLDL VLOVKEKKGY VQNILANNGY VQNILANNGY VQNILANNGY VQNILANNGY VQNILANNGY VQNILANGY VXNI VXNI VXNI VXNI VXNI VXNI VXNI VXNI	DX TAV IWNLD TTI LWDLD TTV LWNLD TTV LWNLD TTV LWNLD TTV LWNLD TTV LWNLD RVI SA SVD PLV LWDVD SF IMUDVD SF IMUDVD GTV FWSVA GTHGWDVDWA KIFGWD FDWG ISAYKQVID ISAYKQVID ISAYKQVID ISAYKQVID ISAYKQVID ISAYKQVID ISAYKQVID ISAYKQVID ISAYKQVID ISAYKQVID ISAYKQVID ISAYKQVID ISAYKQVID ISAYKQVID ISAYKQVID	XD(W/X) TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWAAGSSK TNDWAAGSSK TNDYENN TLDYENN TLDYENN TLDWKHH SLDWKSK FVDWKIN PENWGIAMPA PENWGIAMPA PENWGIAMPA S40 LOOP4 VATCYNISHP VATCMNITPP VATCMNITPP VATCMNITPP VATCMNITPP VATCMNITPP VATCMNITPP	289 303 289 300 278 176 171 162 151 363 3197 215 219 236 355 367 345 355 367 345 243 223
ChDal Chdall McCDA RcCDA GbCDA AnCDA AnCE4A SpPgdA VpCOD VcCOD SbCOD ChDal ChDal Chdall McDA RcCDA RcCDA CbCDA	R(I	DY1AMGTNGT DY1AMGTNGT DY1AMGTNGT SY2EFIQDA DY1AMGTNGT DY1AMGTNGT DY1AMGTNGT CFIQMGNNGS EFIQMGNNGS EKFNNELSAD DYFQLA CFIDQLA	480 TKDFKADGL VTKDFKADGL VTKHFQADGL VSSKLKGDGL FANSGN FATSGQ FATSGQ FATSGQ MASGGN VGANSY GGT GGN	400 1 CATSDLKPW CATSDLKPW CATSDNLKPW LOOP 3	EPGYACDTAN EPGYVCDPAN DPAFACTAET IX IVLT IVLT IVLT IVLT IVLT IVLT IVLT IVLT	420 	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL VRWIATQLNL VRWIATQLNL VRWIATQLNL VRWIATQLNL VLWIATQLNU VLV ANDLDY VLVMILANNGY VQNILANNY VQNILAN	DX TAVIWNLD TTILWDLD TTVLWNLD TTILWDYD HIIDTNLD HVINANID RVISASVD GTVFWSVA GTHGWDVDWA GTHGWDVDWA GTHGWDVDWA KIFGWDFDWG IISAYKQVID IKAAYKNVLD IISAYKQVID IKAAYKNVVD MLKSYKHVVN LKSKGYRAVT	XD(W/X) TDDWAAGTSE TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWAAGDGV TKDYE NN TLDYE HD TKDYE NN TLDYE NN T	289 303 289 300 278 176 171 162 151 363 197 215 219 236 356 370 355 345 243 367 345 2233 226
ChDai ChDai McDA RcDA GbCDA CiCDA PesCDA ArCEA ArCEA SpPgdA VpCOD VcCOD SbCOD ChDai ChDai Chdai McDA RcCDA GbCDA CiCDA PesCDA ArCEA	R(1 	DY1AMGTNGT WRPAFGD WRPAFGD WRPAFGD WRAPYGD WRAPYGD WRAPYGD WRAPYGD WRAPYGV ARLPYTNGWR	480 VTKDFKADGL VTKHFQADGL VSKLKGDGL FANSGN FANSGN FATSGQ MASGGN GGT	400 1 CATSDDLKPW CATSDNLKPW CATSDNVPPW LOOP3	EPGYACDTAN EPGYVCDPAN DPAFACTAET IVLT 	420 I I I VDDR VDDR VDDR VDDR VDDR CDAG I I I I I I I I I I I I I	VRWIASQLGL VRWIASQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL CGGDLGGLGY ALSTLGGLGY VLOVMRDLDY GAVYDRLIPY TDDIRNSLDL VLOKEKKLGY VQNILANNGY IQNMLAESDY IQNMLAESDY -SLAVENLPK -SLAVENLPK -SLAWEYLPK -SLAWEYLPK -SLAWEYLPK VSLTQKLIDT HYLVQEAINA ASLAERMLQE KAVPQ-LVSK	DX TAVIWNLD- TTILWDLD- TTILWDLD- TTVLWNLD- TTILWDYD- HIIDTNLD- HVINANID- RVISASVD- PLVLWDVD- SFIMWDVD- GTVFWSVA- GTHGWDVDWA KIFGWDFDWG IISAYKQVID IRAAYKNVLD IISAYKQVID IISAYCVID	XD(W/X) TDDWSAGVTT TDDWAAGTSE TDDWSAGVTT TDDWAAGSSK TNDWAAGSSK TNDWAAGSSK TNDYENN TLDYENN TLDYENN TLDWKHH SLDWKSK FVDWKIN PENWGIAMPA PENWGIAMPA LOOP4 VATCYNISHP VATCMNITPP VATCMNITPP VATCMNITPP VATCMNITPP VATCMNITPP VATCMAITPP VATCMAITPP	289 303 278 3776 1711 162 151 215 219 236 356 370 355 367 345 243 2233 2243 2233 2243 2236 209
ChDal Chdall MrCDA RcCDA GbCDA ArCEAA SpPgdA VpCOD VcCOD SbCOD ChDal Chdall MrCDA RcCDA GbCDA ClCDA ArCE4A SpPgdA	R(I	DY1AMGTNGT DY1AMGTNGT DY1AMGTNGT DY1AMGTNGT DY1AMGTNGT DY1AMGTNGT DY1AMGTNGT EFIQMGNNGS EKFNNELSAD DY1AMGTNGS EKFNNELSAD DY1AMGTNGT EFIQMGNNGS	VTKDFKADGL VTKHFQADGL VSSKLKGDGL FANSGN	400 1 CATSDDLKPW CATSDNLKPW CATSDNLKPW LOOP 3	EPGYACDTAN EPGYVCDPAN DPAFACTAET IVLT 	420 	VRWIASQLGL VRWIATQLGL VRWIATQLNL VRWIATQLNL VRWIATQLNL VRWIATQLNL CAGDLGGLGY ALSTLGGLGY VLQMRDLDY TDDIRNSLDL VLKETKRLGY VQNILANNGY NALVRVICY VQNILANNGY NALVRVICY VQNILANNGY VQNILANNGY NALVRVICY VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNGY VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNGY VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY NANNA SLAVENUN VQNILANNGY VQNILANNGY NANNA SLAVENUN VQNILANNGY VQNILANNGY NANNA SLAVENUN VQNILANNA VQNILANNA SLAVENUN VQNILANNA VQNILANNA SLAVENUN VQNILANNA VQNILANNA VQNILANNA VQNILANNA VQNILANNA VQNILANNA VQNILANNA VQNILANNA VQNILANNA VQNILANNA VQNILANNA VQNILANNA VQNILANNA VQNI VQNILANNA VQNI VQNILANNA VQNI VQNILANNA VQNILANNA VQNI VQNILANNA VQNI VQNILANNA VQNI VQNILANNA VQNI VQNILANNA VQNI VQNILANNA VQNI VQNILANNA VQNI VQNI VQNILANNA VQNI VQNI VQNI VQNI VQNI VQNI VQNI VQNI	DX TAVIWNLD TTILWDLD TAVIWNLD TTILWDYD TTILWDYD HIIDTNLD HVINANID RVISASVD GTVFWSVA GTHGWDVDWA GTHGWDVDWA GTHGWDVDWA KIFGWDFDWG ISAYKQVID	XD(W/X) TDDWAAGTSE TDDWAAGTSE TDDWAAGSSK TNDWAAGSSK TNDWAAGSSK TNDWA-SSK TNDYE NN TLDYE	289 303 289 300 278 176 171 162 151 363 215 219 236 356 370 355 345 243 367 345 243 2233 226 420
ChDai ChDai McDA RcDA GbCDA CiCDA PesCDA ArCEA SpPgdA VpCOD VcCOD SbCOD ChDai ChDai Chdai McDA RcDA RcDA GbCDA CiCDA PesCDA ArCE4A SpPgdA BsPdaA	R(1	DY1AMGTNGT WRPAFGD WRPAFGD WRPAFGD WRAPYGD WRAPYGD WRAPYGD WRAPYGD WRAPYGV ARLPYTNGWR MUDIA	480 VTKDFKADGL VTKHFQADGL VSKLKGDGL FANSGN FANSGN FATSGQ MASGGN FATSGQ MASGGN GGT GGT	400 1 CATSDLKPW CATSDNLKPW CATSDNVPPW LOOP3	EPGYACDTAN EPGYVCDTAN DPAFACTAET IVLT 	420 	VRWIASQLGL VRWIASQLGL VRWIASQLGL VRWIATQLNL VRWIATQLNL CGGDLGGLGY ALSTLGGLGY VLOVMRDLDY GAVYDRLIPY TDDIRNSLDL VLOVKETKRLGY VQNILANNGY IQNMLAESDY -SLAVENLPK -SLAVENLPK -SLAWENLP	DX TAVIWNLD TTILWDLD TTVLWNLD TTVLWNLD TTVLWNLD TTVLWNLD RVISASVD PLVLWDVD SFIMDVD GTVFWSVA GTHGWDVDWA KIFGWDFDWG KIFGWDFDWG IISAYKQVID IKAAYKNVLD IISAYKQVID IKAAYKNVLD IISAYKQVID IKAAYKNVLD IISAYKQVID IKAAYKNVLD IISAYKQVID IKAAYKNVLD IISAYKQVID IXAYCU	XD(W/X) TDDWSAGVTT TDDWAAGTSE TDDWSAGVTT TDDWAAGSSK TNDWAAGSSK TNDWAAGSSK TNDYENN TLDYENN TLDYENN TLDWKHH SLDWKSK FVDWKIN PENWGIAMPAA PENWGIAMPAA PENWGIAMPAA ILOOP4 S40 VATCYNISHP VATCYNISHP VATCMNITPP VATCMNITPP VATCMNITPP VATCMISHP VATCKANISHP V	289 303 289 300 278 176 151 363 197 215 236 356 370 355 367 345 233 243 2236 209 420 2258
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FIGURE 2 | Multiple sequence alignment of chitin deacetylases ChDal and ChDall from *Mucor circinelloides* IBT-83. Abbreviations: chitin deacetylases from: *Mucor rouxii* MrCDA (Uniprot: P50325), *Rhizopus circinans* RcCDA (Uniprot: A7UMZ0), *Gongronella butleri* GbCDA (Uniprot: Q8J2N6), *Colletotrichum lindemuthianum* CICDA (PDB: 21W0), *Pestolotiopsis* sp. PesCDA (GenBank: APH81274.1), *Aspergillus nidulans* AnCDA (PDB: 2Y8U); CE4 deacetylase from marine environment Arthrobacter sp. ArCE4A (PDB: 5LGC); peptidoglycan deacetylase from *Streptococcus pneumoniae* SpPgdA (PDB: 2C1G); CE4 esterase from *Bacillus subtilis* BsPdaA (PDB: 1W17); chitooligosaccharides deacetylases from *Vibrio parahaemolyticus* VpCOD (PDB: 3WX7), *Vibrio cholerae* VcCOD (PDB: 4NY2), *Shewanella woodyi* SbCOD (GenBank: ABN60929.1).

circinelloides IBT-83 cDNA, cloned and sequenced (Kaczmarek et al., 2016). The multi-sequence comparison of *in silico* translated ChDaI and ChDaII sequences against other members of the CE4 deacetylase family showed high homology in the area of the NodB domain, also called the catalytic domain. The obtained results presented in **Figure 2** indicate the presence of five catalytic motifs MT1-5 crucial for the functioning of enzymes belonging to the CE4 esterase family: Motif1 (MT1) corresponding to the sequence (M/Y)DD; Motif2 (MT2) H(S/T)xxH, Motif3 (MT3) R (P/x) (P/A) (Y/F/R), Motif4 (MT4) DxxD (W/Y) and the Motif5 (MT5) including the residues LxH. The Tyr255(ChDaI)/Phe269(ChDaII) (MT3) together with Asp164(ChDaI)/Asp178(ChDaII) (MT1) and His320(ChDaI)/ His334(ChDaII) (MT5) residues are directly involved in the amide bond hydrolysis reaction (Nakamura et al., 2017; Tuveng et al., 2017). Moreover, highly conserved residues

Asp165(ChDaI)/Asp179(ChDaII) (MT1), His214(ChDaI)/ His228(ChDaII) (MT2) and His218(ChDaI)/His232(ChDaII) (MT2) form the so-called an metal-binding triad that coordinate the metal atoms; typically one of Zn^{2+} , Co^{2+} , Ca^{2+} ; which are co-factors of chitin deacetylases (Zhu et al., 2019). Moreover, the performed multi-sequence comparison allowed for the identification of six loops in the amino acid sequences of ChDaI and ChDaII proteins. Crystallographic studies carried out previously (Andrés et al., 2014) showed that these loops are located in close proximity to the active centre of the enzyme and are involved in "capturing" the substrates subjected to deacetylation reactions.

EXPRESSION AND ENZYMATIC ACTIVITY OF CHITIN DEACETYLASES CHDAI AND CHDAII FORM *M. CIRCINELLOIDES* IBT-83

The multi-sequence comparison of the amino acid sequences of the ChDaI and ChDaII proteins suggest that the genes chdaI and chdaII identified in the filamentous fungi M. circinelloides IBT-83 encode these enzymes exhibiting chitin deacetylase activity. The fungal chitin deacetylases known so far are glycoproteins, for which the post-translational attachment of sugar residues, mainly mannose, is crucial for the proper functioning of enzymes. Chitin deacetylase from Mucor rouxii has been identified as a monomeric protein with a high content of linked mannose residues (Martinou et al., 1993). Another example of a highly glycosylated chitin deacetylase is an enzyme isolated from soil fungi of the genus Mortierella sp. Importantly, the enzymatic deglycosylation performed at this glycoprotein resulted in a complete loss of catalytic activity (Zhao et al., 2011). Our attempts to functionally express the M. circinelloides IBT-83 chda genes in E. coli were also unsuccessful (results not shown). This is most likely due to inability of this prokaryotic host to carry out the necessary post-translonational modifications. Analysis of the amino acid sequences of the ChDaI and ChDaII proteins using bioinformatic tools NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and (http://www.cbs.dtu.dk/services/NetOGlyc/) NetOGlyc 4.0showed that both proteins have numerous sites of potential Nand O-glycosylation. Due to the ability to carry out posttranslational modifications and the simplicity of genetic manipulation, the yeast Pichia pastoris was selected as an efficient host organism for heterologous gene expression. The open reading frames ORF (without native signal sequences, 21 aa.) encoding ChDaI and ChDaII were successfully cloned and transformed into Pichia pastoris KM71H for and targeted for extracellular expression. The theoretical mass of recombinant proteins enriched with the C-terminal 6xHis tag and Myc Tag was 50.8 and 51.7 kDa for ChDaI and ChDaII, respectively. Figure 3 shows the results of electrophoretic analysis (SDS-PAGE) of proteins present in the culture medium obtained after culturing selected KM71H pPICZ chdaI and KM71H pPICZ chdaII transformants expressing proteins, ChDaI and ChDaII, respectively. Electrophoretic analysis showed that the mass of deacetylase I-ChDaI is approx. 48 kDa, while the mass of the

second expressed protein - ChDaII is approx. 49 kDa. It is surprising that the actual molecular weight of the obtained recombinant chitin deacetvlases from M. circinelloides IBT-83 are smaller than the determined theoretical weight of the proteins. However, it should be remembered that the migration of proteins in the SDS environment, in addition to size, may also be dependent on the structural properties of proteins themselves, their charge, and the ability to bind SDS while forming micelles. The prior research showed that for membrane proteins, the difference between the theoretical mass and the experimental mass determined during SDS-PAGE analysis could be as high as 48% (Rath et al., 2009). Moreover, P. pastoris yeast produces numerous extracellular proteolytic enzymes responsible for the hydrolysis of peptide bonds from the N-terminal end of proteins (Sinha et al., 2005). However, peptide mapping analysis performed on proteins extracted from the gel bands indicated that the recombinant proteins were chitin deacetylases.

The analysis of the enzymatic activity of chitin deacetylases, carried out for the culture medium from different time points of cultivation, showed that deacetylase II has 74% higher maximum specific activity compared to deacetylase I. Figure 4 shows the dependence of the specific activity of ChDaI and ChDaII on the duration of the methanol-induced culture. Both enzymes showed the highest specific activity after 48 h of cultivation, which was 220.47 mU mg⁻¹ of protein and 836.12 mU mg⁻¹ for ChDaI and ChDaII, respectively. The specific activity of chitin deacetylase II adjusted for the protein concentration determined on the basis of the band intensity corresponding to the recombinant protein (Figure 3) was 3.57 U mg^{-1} (Supplementary Figure S4). The analysis of the products obtained as a result of the ChDaII activity, showed a significant increase in the degree of polymer deacetylation (DD). The average DD of the products was 96.0 \pm 1.6% in relation to the substrate, the degree of deacetylation of which was DD 78.13 \pm 0.14%. Since deacetylase II showed a much higher catalytic activity compared to deacetylase I, only isoform II of chitin deacetylase from Mucor circinelloides IBT-83 was subjected to further analyses.

Analysis of the biochemical properties of the recombinant chitin deacetylase from Mucor circinelloides IBT-83 allowed the determination of the optimal temperature for the action of the recombinant enzyme. The highest activity of ChDaII was determined for the reaction temperature of 50°C. Exceeding this value results in a drastic decrease in activity by approx. 90% for the temperature of 60°C and almost complete loss of ChDaII catalytic activity at 70°C (Table 1). Most fungal chitin deacetylases studied so far show the highest catalytic activity at 50°C. However, chitin deacetylases with distinct properties are also known. An example is chitin deacetylase from Rhizopus circinans, for which the optimal temperature was 37°C (Gauthier et al., 2008). On the other hand, native deacetylase from C. lindemuthianum showed higher thermostability and the highest activity at 60°C (Tokuyasu et al., 1996).

Chitin deacetylases, including those of fungal origin, belong to the group of metalloenzymes. Divalent metal ions act as enzyme cofactors that are coordinated by side groups of amino acids







belonging to the metal-binding triad (**Figure 2**). **Table 2** shows the results of the percentage change in the enzymatic activity of recombinant chitin deacetylase II from *M. circinelloides* IBT-83 for reactions carried out in a medium enriched with divalent metal ions at a concentration of 1 mM or 10 mM, compared to the control for which the reaction medium was not enriched with metal ions. The obtained results clearly indicate a high dependence of the recombinant enzyme activity on the type and concentration of bivalent ions. The use of Cu^{2+} and Ni^{2+} ions significantly inhibited the activity of the tested enzyme by 44–94%. For Fe²⁺ ions, a decrease in activity was observed only at the concentration of metal ions at 10 mM. The enzyme activity

TABLE 1 Effect of reaction temperature on the activity of recombinant chitin deacetylase ChDall from *Mucor circinelloides* IBT-83. The culture medium obtained after 48 h of cultivation of the selected transformant was used as the enzyme preparation.

ChDall activity (mU ml ⁻¹)		
34.6		
171.3		
212.7		
288.5		
29.8		
1.9		

TABLE 2 Percentage change in enzymatic activity of recombinant chitin deacetylase ChDall from *Mucor circinelloides* IBT-83. The culture medium obtained after 48 h of cultivation of the selected transformant was used as the enzyme preparation.

Metal ions	Percentage cha activ	nge in enzymatic ity (%)
	1 mM	10 mM
Control	100	100
Cu ²⁺	56	11
Fe ²⁺	98	80
Mg ²⁺	119	112
Mn ²⁺	109	98
Zn ²⁺	135	79
Ni ²⁺	12	6

increased in the presence of Mg^{2+} ions at concentrations of 1 and 10 mM and Mn²⁺ ions at a concentration of 1 mM. Interesting results were observed using Zn²⁺ ions as the enzyme cofactor. For the concentration of 1 mM, an increase in chitin deacetylase activity as much as 35% was observed. Increasing the concentration of metal ions to 10 mM resulted in a decrease in activity by 21%. Literature data show that the effect of individual metal ions on the activity of chitin deacetylases isolated from various microorganisms, mainly fungi, vary from protein to protein and it is difficult to determine the direct relationship between the amino acid sequence of proteins and their response to the presence of individual metal ions in the reaction environment. The closely related, native chitin deacetylase from M. rouxii (MrCDA, Figure 2) showed increased activity after incubation in a buffer enriched with Zn^{2+} ions (1 mM), while the use of Mn^{2+} ions inhibited the biocatalyst (Kołodziejska et al., 1999). On the other hand, both C. lindemuthianum chitin deacetylase (ClCDA) and ChDaII were significantly inhibited by Ni²⁺ ions despite significant sequence differences within the catalytic motifs. Furthermore, Mn²⁺ ions at a concentration of 10 mM limited only the activity of native ClCDA (Tokuyasu et al., 1996). Moreover, native and recombinant enzymes derived from the same microorganisms may also show different effects of metal ions on their activity. The previous studies showed that the activity of the native chitin deacetylase from Rhizopus circinans is increased in the presence

of Mn^{2+} and Mg^{2+} ions (1 mM) and was strongly inhibited by Cu^{2+} ions (Gauthier et al., 2008). The recombinant enzyme showed no significant changes in activity due to the presence of manganese and magnesium ions, however, like the native protein, it was strongly inhibited by Cu^{2+} ions. Chitin deacetylase from *C. lindemuthianum* expressed in *P. pastoris* also showed different properties than the native enzyme (Blair et al., 2006).

Polycistronic Expression Vector Synthesis

The factor limiting the use of enzymatic methods in large-scale modification of chitin substrates is the high crystalline structure of the polymer. Crystallinity limits the availability of reactive groups present in the polymer chains for enzyme action. This phenomenon is often observed in case of chitin deacetylases (Pareek et al., 2013). Moreover, the bioconversion of chitin substrates to chitooligosaccharides with the desired chain arrangement requires the action of several enzyme preparations, which increases costs of these processes. The currently used conventional thermo-chemical and chemical methods have many disadvantages, with uncontrolled degradation of polymers as the most important one. The latter results in a mixture of heterogeneous products (Struszczyk, 2000). Nevertheless, high efficiency and low costs mean that they are still used in industrial processes. It is expected that simultaneous expression of three genes encoding proteins with chitin- and chitosanolytic activity under the control of one promoter might increase the efficiency of enzymatic processes and reduce the costs associated with the production of biocatalysts. A polycistronic expression vector incorporating the genes of chitin deacetylase ChDaII, chitinase from Thermomyces lanuginosus, and chitosanases from Aspergillus fumigatus was prepared employing the LCR method. Ligase cycled reaction is an efficient and powerful tool to assemble many DNA fragments in a single reaction. The selections of genes encoding chitinase and chitosanase were based on the results of biochemical analysis of recombinant chitin deacetylase ChDaII from Mucor circinelloides IBT-83. Analysis of the literature data showed that the best candidate for heterologous expression of chitinase was chitinase1 (Chit1) from Thermomyces lanuginosus, which was already heterologously expressed in P. pastoris GS115 strain. The optimal temperature for this enzyme was 50°C, while the optimal pH of the reaction medium was pH 5.0 (Zhang et al., 2015). The advantage of this biocatalyst, compared to the other candidates, was that it exhibits satisfactory exo- and endochitinolytic activity against native chitin derived from powdered shrimp shells. The selected chitosanase from Aspergillus fumigatus was also successfully heterologously compressed in P. pastoris GS115. The recombinant enzyme showed high activity over a wide pH range from 4 to 7. The optimal temperature of its activity was 60°C, however, at 50°C it showed catalytic activity exceeding 80% of the maximum activity. Like native ChDaII, the chitosanase from A. fumigatus contained an N-terminal signal sequence of 17 amino acids (Chen et al., 2012). To avoid potential problems with the



presence of the native signal peptide, a gene lacking this sequence was used for the preparation of the polycistronic vector pCHIT. The genes coding each enzyme were separated by two different A2 self-processing sequences. Verification of the assembly was performed with a series of PCR reactions using appropriate primers (Supplementary Table S3). Figure 5A shows the results of the electrophoretic analysis of PCR reaction products. The efficiency of assembling six DNA fragments, the size of which ranged from 50 bp to 3,500 bp, was 50%. Prior research showed that the LCR method allows for the assembly of eight fragments with efficiency reaching even 90%. (Roth et al., 2014). For each of the three enzymes a-factor secretion signal was used to guide the secretion of recombinant proteins. This approach was used to ensure a similar efficiency of expression and secretion of individual proteins. The degeneracy of the genetic code was used in the optimisation of secretory sequences to reduce the risk of homologous recombination between the enzymes and subsequent loss of function. Despite these efforts, these sequences showed a relatively high homology, which could have exerted an impact on the correct synthesis of the pCHIT vector. Similar sequence homology problems were observed for the viral A2 selfprocessing sequences. These sequences show little interspecies variability, which can also impede the correct synthesis of the polycistronic vector pCHIT. In the light of these sequence homology challenges, the efficiency of the process reaching 50% can be considered a success. De Kok et al. (2014) proved that a similar efficiency of DNA fragment assembly could be obtained only with the use of yeast recombination mechanisms. However, LCR assembly

provides a much faster and easier workflow than recombination *in vivo*.

Expression of Three Enzymes From the Polycistronic Vector

The correctly synthesised polycistronic expression vector was linearised with SacI and electroporated into Pichia pastoris KM71H cells. Selected transformants, for which integration of the expression vector into the host genome was confirmed by colony PCR, were subjected to methanol-induced heterologous expression. Figure 6 shows the results of electrophoretic analysis (SDS-PAGE) of proteins present in the culture medium obtained after culturing the selected KM71H_pCHIT transformant. The gels show three biosynthetic products, the mass of which ranged from 46 to 48 kDa for product 1 and 2 (chitin deacetylase ChDaII from Mucor circinelloides IBT-83 and chitinase from T. lanuginosus, enriched with their A2 self-processing sequences, both respectively), and about 27 kDa for product 3 (chitosanase from A. fumigatus). According to the mechanism of functioning of A2 selfprocessing sequences, the gene preceding the viral sequence will be enriched with the N-terminal fragment of these sequences. Therefore, the theoretical mass of chitin deacetylase II from M. circinelloides IBT-83 enriched with an 18 amino acid fragment of P2A sequence was 49 kDa, while the predicted mass of recombinant chitinase from T. lanuginosus with a 17 amino acid N-terminal fragment of the T2A sequence was 45.9 kDa. The obtained products 1 and 2 (Figure 6) correspond to ChDaII + P2A chitin deacetylase and T. lanuginosus chitinase enriched with T2A sequences. As it was mentioned, chitinase from T. lanuginosus was already expressed in P. pastoris GS115 strain, and its mass was 44.1 kDa. However, this variant was not enriched



with the T2A viral sequence (Zhang et al., 2015). The weight of the third product (27 kDa, **Figure 6**) differs from the theoretical weight of chitosanase from *A. fumigatus*, which was 23.6 kDa. The results earlier showed that the recombinant chitosanase from *A. fumigatus* has a mass of approx. 28 kDa when expressed in *P. pastoris* (Chen et al., 2012). Most likely, it proves a relatively high protein glycosylation (approx. 18.6% in relation to the protein mass). Bioinformatic analysis showed that this enzyme has several potential O-linked glycosylation sites and one potential N-linked glycosylation site. Moreover, it may be related to the process of cleavage of the secretory sequence by the native KEX2 proteinase produced by the yeast *P. pastoris* (Kjeldsen, 2000). The theoretical mass of chitosanase, taking into account the secretory sequence, is approx. 32 kDa.

Recombinant proteins extracted from the two electrophoretic bands were subjected to peptide mapping analysis. Trypsin digestion was performed with proteins extracted from the upper band potentially containing *M. circinelloides* IBT-83 chitin deacetylase and *T. lanuginosus* chitinase and the lower band containing *A. fumigatus* chitosanase. The obtained peptides (from the upper band) were aligned with the *T. lanuginosus* chitinase sequence (GenBank accession number AIW06013.1) and the *M. circinelloides* f. *lusitanicus* CBS 277.49 chitin deacetylase sequence (GenBank accession number OAD01792.1). Protein sequence coverage was 62 and 6%, respectively, for recombinant chitinase from *T. lanuginosus* (Supplementary Figure S1) and ChDaII from *M. circinelloides* IBT-83 (Supplementary Figure S2). Peptides obtained after digestion of the lower band protein were aligned with the endochitinase sequence from *Asperillus* sp. CJ22-326 (GenBank accession number ABZ88800.1) with the protein sequence coverage at 39% (Supplementary Figure S3). The obtained results clearly indicated the presence of recombinant proteins in the culture medium confirming the correct functioning of the A2 self-processing viral sequences.

Viral self-processing A2 sequences have been successfully used for multi-gene polycistronic expression in cells of various eukaryotes, incl. human and mouse cell lines, plants and yeast cells (Geier et al., 2015). The advantage of using A2 sequences for multi-gene expression is their small size compared to IRES (Internal Ribosome Entry Sites) sequences, which significantly reduces the size of multi-gene constructs, and thus facilitates their transformation into efficient producer organisms. Early studies showed that the amount of proteins obtained from the expression of genes upstream and downstream of A2 sequences is the same or very similar (Halpin et al., 1999). This was supposed to be another advantage over IRES sequences. Subsequent in vitro analysis of the translation process showed some disproportions in the amount of proteins expressed using the A2 sequence. De Felipe et al. (De Felipe et al., 2006) showed that proteins encoded ahead of A2 sequences are produced in a slightly higher amount than proteins that genes are located behind them. These differences, however, were not as significant as when the IRES sequence was used. Our results may indicate that the expression efficiency of such polycistronic systems also depends on the size of the expressed genes. The band indicating the presence of chitosanase, the smallest and the third of the three co-expressed genes, appeared in the culture medium in the highest amounts (Figure 6). It is unclear at this stage as to whether this difference is due to translation, folding or protein export.

ENZYMATIC ACTIVITY OF THE MULTI-ENZYME COCKTAIL

The culture medium obtained after 96 h of methanol-induced cultivation of P. pastoris KM71H_pCHIT strain was analysed for the activity of chitin deacetylase, chitinase and chitosanase Table 3). The activity of recombinant chitin deacetylase was 372 mU mg⁻¹ of protein. However, taking into account the intensity of the band peptide identified as chitin deacetylase II shown in Figure 6, the adjusted specific activity of the recombinant enzyme was $3.97 \text{ U} \text{ mg}^{-1}$ (Table 3; Supplementary Figure S4). The obtained results indicate that the specific activity of the enzyme expressed in the multigene system is slightly higher than that of ChDaII after expression of the monogenic vector. This may indicate the synergistic action of biocatalysts in the multi-enzyme cocktail. Pre-hydrolysis of the glycosidic bonds loosens the dense structure of the chitin substrate, thereby increasing the availability of acetyl residues for the action of chitin deacetylase. The degree of deacetylation of the products obtained by the action of ChDaII chitin deacetylase from the multigene system reached $85.22 \pm 0.17\%$.

Enzyme	Substrate	Protein concentration (mg ml ⁻¹)	Activity (U ml ⁻¹)	Specific activity (U mg ^{−1})	Adjusted protein concentration (mg ml ⁻¹) ^a	Adjusted specific activity (U mg ⁻¹) ^a
Chitin deacetylase	0.5% chitosan ^b	0.68	0.25	0.37	0.063	3.97
Chitinase	Colloidal chitin		1.99	2.93	0.039	51.03
Chitosanase	0.5% chitosan ^c		3.46	5.09	0.295	11.73

TABLE 3 | Enzymatic activity determined in the post-culture liquid obtained after expression of the pCHIT transformants.

^aDetermined on the basis of the intensity of the band corresponding to the recombinant protein.

^bDissolved in hydrochloric acid.

^cDissolved in 2% acetic acid.

The activities of recombinant chitinase and chitosanase were determined by measuring the concentration of reducing sugars. Since both enzymes were present in the culture medium used as the enzyme preparation, it was assumed that recombinant chitinase from T. lanuginosus shows activity against colloidal chitin, while recombinant chitosanase from A. fumigatus was active only against chitosan dissolved in 2% (v/v) acetic acid. However, it should be remembered that acetylated (GlcNAc) and deacetylated (GlcN) residues occur in both chitin and chitosan and can be hydrolysed by both enzymes. Determined in this way, the specific activity of the recombinant chitinase was 2.9 U mg⁻¹. Determined based on densitometric analysis of the SDS gel (Figure 6; Supplementary Figure S4), the concentration of recombinant chitinase was 63 µg ml⁻¹, which allowed to determine the adjusted specific activity of 51.03 U mg⁻¹. Recombinant chitinase from *T. lanuginosus* expressed in P. pastoris GS115 in addition to activity against synthetic substrates such as: 4-nitrophenyl N,N'-diacetyl-β-Dchitobioside (exochitinolytic, 30 mU mg⁻¹) and 4-nitrophenyl β-D-N,N',N"- triacetylchitotriose (endochitinolytic, 150 mU mg⁻¹), also showed activity against colloidal chitin. However, this value was not disclosed (Zhang et al., 2015). Chitinase from Bacillus licheniformis expressed in P. pastoris KM71H yeast showed a specific activity of 3.4 mU mg⁻¹ protein in the culture medium (Menghiu et al., 2019). On the other hand, chitinase from Streptomyces albolongus ATCC 27414 expressed in E. coli BL21 bacteria showed specific activity against colloidal chitin of as much as 66.2 Umg⁻¹ of protein (Guo et al., 2019). This indicates the wide range of activities that biocatalysts from various sources exhibit.

The activity of the third enzyme expressed in the polycistronic system pCHIT was 5.2 U mg⁻¹ against 0.5% (w/v) of chitosan dissolved in acetic acid. After taking into account the concentration of recombinant chitosanase in the after-culture medium (determined on the basis of the intensity of the band), the corrected specific activity was 11.73 U mg⁻¹ (Table 3; Supplementary Figure S4). Previous studies focused on the expression of this enzyme in the yeast P. pastoris GS115 at the bioreactor scale of (Chen et al., 2012). The pre-purified recombinant chitosanase obtained from A. fumigatus showed a specific activity of 8.3 U mg⁻¹. The higher activity of the enzyme obtained in our research may be due to the fact that recombinant chitinase hydrolysing glycosidic bonds on acetylated units was also present in the reaction mixture. The chitosan used as a substrate for the determination of chitosanase activity showed a deacetylation degree of 78.13 \pm 0.14%. This indicates that acetylated units susceptible to

the action of recombinant chitinase were present in the substrate. The ability of the multi-enzyme cocktail to modify colloidal chitin was confirmed by GPC/SEC analysis of products obtained as a result of the action of recombinant proteins present in the after-culture medium (**Supplementary Figure S5**). The number-average (Mn) and weight-average (Mw) molar masses of the substrate and the obtained products determined on the basis of the obtained results clearly indicate the occurrence of the enzymatic depolymerization of colloidal chitin (**Supplementary Table S4**). Meanwhile the polydispersity index ~1.0 indicates that there is a high degree of homogeneity among these degradation products suggesting that chitooligosaccharides of similar chain length have been synthesized. Unfortunately, the set of columns used in the analysis did not allow to accurately determine the masses of the products obtained during enzymatic degradation of the biopolymer.

To the best of our knowledge, our research is the first attempt at a comprehensive, multi-enzymatic modification of chitin substrates using a mixture of several enzymes belonging to different classes and produced by one host. Chyleński et al. used Design of Experiment (DoE) approach to create highly efficient enzyme cocktails for the complete enzymatic hydrolysis of lignocellulosic biomass (Chylenski et al., 2017). Two-stage enzymatic hydrolysis was carried out using five different enzyme mixture components. The chitin-rich biomass was also subjected to a total saccharification process using enzymatic cocktail. The DoE strategy included the use of five mono-component enzymes from Serratia marcescens, three chitinases, SmChiA, SmChiB, SmChiC, a lytic polysaccharide monooxygenase, SmLPMO10A, and a β-N-acetylhexosaminidase (SmCHB). The enzymes were heterologously expressed in monogenic E. coli expression systems. The optimised enzyme cocktail resulted in the saccharification degree of shrimp and crab chitins of 70-75%. Meanwhile, a "minimal" cocktail yielded only 40% saccharification (Mekasha et al., 2017). The increase of yield affirms the hypothesis that chitinolytic enzymes interact synergistically with each other in processing chitin and its derivatives. Of course, the DoE approach assumes a wider optimisation of the reaction conditions (enzymes, mix ratios, temperature, pH, salt, and cofactors), but it does not assume the simultaneous production of biocatalysts in one host. Therefore, the costs of the processes are multiplied by the necessity to produce and purify recombinant enzymes that are involved in multi-enzyme cocktails.

Our research has shown that the use of polycistronic expression of chitin- and chitosanolytic enzymes using self-processing A2

sequences has a real chance to overcome these limitations. Moreover, the use of efficient *P. pastoris* expression systems that produce small amounts of native extracellular proteins allows the application of raw culture medium as enzyme preparations significantly facilitating the recombinant proteins purification. However, careful examinations of the relationships between the efficiency of the expression of individual genes, their location in the multi-gene expression cassette, gene properties (size, spatial protein structure) and helper sequences (secretory sequences, A2 sequences) are still necessary.

CONCLUSION

In this work we have expressed and characterised two chitin deacetylases from *M. circinelloides* IBT-83 and utilised one of these proteins for the synthesis of the first eukaryotic, polycistronic expression system employing self-processing 2A sequences containing three sequentially acting chitin processing enzymes. During the study it was shown that using viral self-processing sequences and degeneracy of the genetic code it is possible to stably express synergistically acting extracellular biocatalysts under control of the same promoter. These findings will hopefully translate into more effective conversion of chitin waste into their valuable derivatives: chitosan and chitooligosaccharides.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author.

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AUTHOR CONTRIBUTIONS

MBK: Conceptualization; Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing - original draft, Writing - review and editing; KS-S: Conceptualization; Methodology; MX: Investigation, Data curation, Visualization; MS-A: Conceptualization, Funding acquisition, Methodology, administration, Project Resources, Supervision; TA: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision; MG: Data curation, Formal analysis, Investigation, Methodology, Validation; AS: Writing - review and editing; MD: Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Software, Supervision, Visualization, Writing - review and editing.

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SUPPLEMENTARY MATERIAL

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