Molecular Cloning, Over-expression, Kinetic and Structural Properties of Purified Recombinant Family VII Carboxyl esterases

T. Ndiitwani, KJ. Rashamuse, SW. Limani, MB. Nthangeni, MG. Tlou

***Abstract*—** Carboxyl esterases (EC 3.1.1.1) are α/β hydrolases which act on acylglycerols to liberate fatty acids and glycerol. They are amongst the most sort-after biocatalysts due to the potential for application in various industries i.e. food, dairy, pharmaceutical, detergents, textile, biodiesel, and cosmetic industries, a factor that drives the continued identification and kinetic characterization for these enzymes. The carboxyl esterase (CEST) genes from *Geobacillus kaustophilus (*Est179), *B. licheniformis* (Est96) and *B. pumilus* (Est95) were expressed in *Escherichia coli.* For the purified CESTs, temperature optima assays showed that Est95 and 96 were optimally active at 45^{o}C while the optimum activity for Est179 was recorded between 45^{o}C and 55 ^{o}C. Furthermore, Est95 and 96 retained more than 95% of activity at temperature ranges between 25 – 40 ° C while, Est179 retained about 80-100% activity between 25-60 ^{o}C. The CESTS were also subjected to substrate specificity assays which revealed that the enzymes showed high affinity for *p*-nitrophenyl butyrate (ρNP-C4) as demonstrated by the low K_m obtained in comparison to other test substrates. However, the ratio for the specificity constants (k_{cat}/K_M) revealed ρNP–C3 (propionate) was a preferred substrate for Est95, while ρNP–C2 (acetate) and ρNP–C4 were preferred substrates for Est96 and Est179, respectively. Circular Dichroism (CD) spectroscopy-assisted secondary structure predictions recorded spectra that were consistent with a α-helical content for all three proteins. Intrinsic fluorescence spectroscopy-based comparative analysis of protein tertiary structure revealed a difference in the conformation for the 3 proteins, a possible explanation for the differences in the kinetic properties. Therefore, this study constitutes a report on three family VII CESTs that are optimally active at near-neutral to alkaline pH (6.5 – 9), mesotable to moderately thermostable and with a preference for short-chain acyl esters. The outcomes of this study forms the basis for future experiments aimed at evaluating CESTs properties for potential application in specific industries.

Keyword: Bacillus species**, Family VII** Carboxyl esterases, enzyme kinetics, intrinsic fluorescent and Circular Dichroism Spectroscopy.

I. Introduction

Lipolytic enzymes are carboxylic ester hydrolases which act on acylglycerols to liberate fatty acids and glycerol. These enzymes have been grouped into two major classes based on substrate specificity, carboxyl esterases (3.1.1.1.) and lipases (EC 3.1.1.3), which respectively hydrolyse esters of short-chain carboxylic acids (C < 10) and maximally active towards water insoluble long chain substrates (C ≥ 10) [1, 2]. Although lipolytic enzymes have been found in many species of animals, plants, bacteria, yeast and fungi, the enzymes from microorganisms have attracted considerable attention because of their potential applications in various industries such as food, dairy, pharmaceutical, detergents, textile, biodiesel, and cosmetic industries [3].

Since the classification of bacterial lipolytic enzymes into 8 families by Arpigny and co-workers [4], more enzymes have been identified and the classification recently updated to 19 families [2]. The choice enzymes for this study are classified under the Family VII, which is characterized by enzymes that are relatively large in size (~55 kDa) which shared significant homology (30% identity, 40% similarity) with eukaryotic acetylcholine esterases and mammalian CESTs. This family was previously reported to be comprising of a few enzyme by Arpigny and co-workers [4] and since then, more than a 100 putative Family VII bacterial CEST could be revealed through analyses of nucleotide sequences deposited in nucleotide databases. Most of the putative Family VII gene sequences are discovered as part of genome DNA sequences of microorganisms from extreme environments, organisms that are deemed to be of industrial importance or that have health related concerns. The proteins showing significant similarity (more than 30% amino acid sequence identity) with the reference Family VII *Arthrobacter oxydans* CEST include proteins identified in *Salmonella*, *Mycobacteriaceae*, *Bacillaceae*, *Streptosporangium*, *Thermomonospora*, *Thermobifida* and *Desulfitobacterium* species.

To date, there are relatively few reports on the expression and biochemical characterization of these enzymes which suggests that the biotechnological potential is underexplored. The biotechnological potential for this group enzymes is supported by the identification of Family VII CESTs that are active at elevated temperatures [5], catalyze the entatioselective resolution of racemic drug intermediates [6], biodegradation of the pesticide, carbamate [2] and various other properties [7; 8]. This therefore, necessitates studies aimed at investigating the various reactions that are characteristic to this family of enzyme and a comparison of function to protein structure.

The three dimensional structures of p-nitrobenzyl esterase from *B. subtilis* [9], and *G. stearothemophilus* [10] which are members of Family VII lipolytic proteins have been determined. The structural analyses of proteins revealed that the proteins belong to the alpha/beta hydrolase family [11]. The proteins contain a catalytic triad composed of Ser, His and Glu and as with the mammalian CEST they fold into three structural

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T. Ndiitwani and MG. Tlou: Department of Biochemistry, School of Physical and Chemical Sciences, Faculty of Natural and Agricultural Sciences, North-West University, Mafikeng Campus, MMabatho 2790, South Africa

KJ. Rashamuse: Council for Scientific and Industrial Research, Biosciences, P.O. Box 395, Pretoria, 0001, South Africa

SW. Limani and MB. Nthangeni:Onderstepoort Biological Products, Old Soutpan Road, Onderstepoort, Pretoria 0001, South Africa.

domains, a catalytic domain, an alpha/beta domain and a regulatory domain [10]. The availability of the three dimensional structure of *G. stearothermophilus* CEST has enabled molecular dynamics simulation studies to identify thermostable and thermosensitive regions of this member of Family VII lipolytic protein [12]. However, the availability of technologies such as Far-UV Circular Dichroism (CD) [13] and Intrinsic Fluorescence Spectroscopy [14], respectively allow for the preliminary analysis of protein secondary and tertiary structure which can complement the intricacies of protein molecular modelling and simulations.

We therefore, report in this study the utility of gene codon adaptation in the expression of a putative CEST belonging to Family VII lipolytic proteins identified within the thermophilic *G. kaustophilus* genome, the expression of mesophilic CEST derived from *B. licheniformis* and *B. pumilus* and the biochemical properties of the three enzymes. We also report on the Intrinsic Flourescence and Circular Dichroism spectroscopy – based comparative analysis of the secondary and tertiary structures for the proteins. The findings reported in this study will form the basis for future application-based investigations for this family of enzymes.

II. Materials And Methods

A. Dna Manipulations, Bacterial Strains, And Culture Conditions

Bacillus pumilus MBB02 [15], *B. licheniformis* DSM12369 and *G. kaustophilus* HTA426 (obtainable from the Japanese Collection of Cultures) were used. *Escherichia coli* JM109 and *E. coli* JM109 DE3 host and expression cells were purchased from Promega (Madison, USA). The expression plasmid pET28a was obtained from Novagen. The bacterial strains were grown in Luria Bertani (LB) media [20] at 37 ^{o}C with shaking in 5ml, 50 ml or 250 ml shake flasks. $CaCl_2$ competent *E. coli* cells were prepared and transformed with DNA as described by [16].

1. Dna Techniques

Standard microbial and recombinant techniques using commercially available molecular biology grade enzymes were as described by [16]. Oligonucleotide primers were purchased from Intergrated DNA Technologies (USA). The DNA fragments for subcloning and plasmid DNA (pGemT-easy and pET28a) were recovered and purified GFX PCR and Gel band purification kit and Biospin Plasmid DNA extraction kit, respectively. Sequencing was done at Inqaba using the T7 and Sp6 promoter primers. The nucleotide sequences were translated to amino acid sequences and analysed using the Expasy Proteomics tools (http://www.expasy.ch).

B. Cloning Of Carboxyl Esterase Genes

The genomic DNA sample *B. licheniformis* and *B. pumilus* was used as the template using the forward and reverse primer pairs indicted in supplementary Table 1. The PCR was done according to standard conditions using Expand DNA polymerase (Roche).

The open reading frame encoding the CEST gene from *G. kaustophilus* was also codon optimized for expression in *E. coli* and synthesized by GeneArt (Germany). The sequence of the *G. kautophilus* was optimized for codon adaptation in *E. coli*. The gene was re-synthesized chemically (GeneArt, Germany) and subcloned into pUC18 to create pGA-Gk-CEST. The re-synthesized gene contained the *Nco*I and *Xho*I restriction sites on the 5 and 3 prime ends, respectively. The incorporation of the restriction sites, as can also be seen in supplementary Table 1, is to facilitate directional cloning into expression vector pET28a.

C. Expression Of Carboxyl Esterases

The CEST genes were released from pGemT-Easy/pGA-Gk-Cest vector using *Nco*I and *Sal*I and ligated to pET28a digested with *Nco*I and *Xho*1 to create plasmids denoted MBE179, MBE95 and MBE96 corresponding to pET28a vector carrying carboxyl esterase CEST genes from *G. kaustophilus*, *B. licheniformis* and *B. pumilus*, respectively. All the plasmids were transformed into *E. coli* JM109 (DE3) cells and a single colony (from each transformation) containing the plasmid was grown in 50 ml LB containing kanamycin to anabsorbance of approximately 0.8 at 600 nm. The culture was then adjusted to0.5 mM IPTG, and incubation continued at 30 ^{o}C for 10h. Cells were harvested by centrifugation at 5000 X g for 10 min, resuspended and Incubated in 10 mM phosphate buffer, pH 8.0 containing 1mg/ml lysozyme for 30 min, followed by sonification with a Branson Sonifier Cell Disrupter to release intracellular proteins. The cell-free extract was centrifuged at 10 000 g for 20 min to remove cell debris, and assayed for carboxyl esteraseCEST activity. Fractions containing intracellular and extracellular lipase activity were combined, lyophilized and stored at -20 ^{o}C.

D. Purification Of Recombinant Carboxyl Esterase Genes

Following lypholization crude enzyme extracts were dissolved in 50 ml of Na-phosphate buffer (20 mM, pH 7.4) and were loaded onto a HisPrep^{TM} 16/10 IMAC Ni-Sepharose 6 Fast Flow column (Amersham Biosciences) pre-equilibrated with Na-phosphate buffer (20 mM, pH 7.4) containing 0.3 M NaCl and 20 mM Imidazole.(Wash buffer). Unbound proteins were washed with 5 column volumes of wash buffer. Proteins bound to Ni-Sepharose matrix were eluted over 5 column volumes at a flow rate of 5 ml min^{-1} with a one-step gradient of 0.5M imadazole generated with Na-phosphate buffer (20 mM, pH 7.4) containing 0.3 M NaCl and 0.5 M Imidazole. Fractions (5 ml) were collected and the imidazole was removed by dialysis and fractions exhibiting activity were pooled, concentrated (Centriprep YM-10, cut-off, 10 kDa, Millipore) before analysed on the SDS-PAGE.

E. Protein And Enzyme Activity Assays

For routine enzyme assay, esterase activity was determined as essentially described by [17]. Protein concentrations were determined by the method of [18], using bovine serum albumin (BSA, Sigma-Aldrich) as a standard. Denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of [19].

1. Effect Of Ph And Temperature

Thermostability of the purified esterases was determined over the temperature range of 25-90 ^{o}C. Enzyme samples were incubated for 10 min at various temperature ranges in Tris-HCl (50 mM. pH 7.5) and the residual activity determined using standard assay.

2. Kinetic Constants

Experimental data of initial velocity versus substrate concentration with coefficients of variation of <5% were fitted to the Michaelis-Menten equation by weighted nonlinear regression (Hyperbola) analysis using GraphPad Prism version 5.0.

F. Far-Uv Cd And Intrinsic Spectroscopy

Far-ultraviolet circular dichroism (far-UV CD) spectra were measured between 250 and 180 nm in 2 mm quarts cuvette at 25°C using a Jasco J-500 spectrophotometer (Jasco, China) equipped with a temperature control system. The CD spectra are an average of 15 accumulations, using the scan speed of 100 nm/min,1 nm bandwidth, 0.5 nm data pitches were applied, with response time of 1 s. The concentration of hybrid and parental CESTs was 5 µM in 10 mM sodium phosphate buffer, pH 7.4. The spectra were buffer corrected and converted to mean residue ellipticity [θ] using the following equation: [θ] = (100θ)/(cnl)[θ] is the molar ellipticity (degrees square centimetre perdecimole), θ is ellipticity (millidegrees), c is the protein concentration (millimolar), n is the number of residues in the peptide, and l is the path length (centimetres). Analysis of secondary structural content of CESTs was performed using the online web server DichroWeb (http://www.cryst.bbk.ac.uk/cdweb).

Fluorescence spectrophotometer was used to assess localized conformational changes in the tertiary structure of enzymes. The Trp and Tyr residues were both excited at 280 nm. Emission spectra were measured between 290 and 450 nm using a Jasco FP-6300 spectrophotometer (Jasco, china) at 20 °C. The scan speed of 100 nm/min and emission bandwidth of 2.5 nm were used. Excitation and emission slits were both set at 2.5 nm. The assays were carried out in 1 cm sealed cell using 5 µM of each protein in 10 mM sodium phosphate buffer, pH 7.4. The spectra are an average of three accumulations.

III. RESULTS

A. *Carboxyl Esterase Genes Cloning And Optimisation*

The DNA sequences containing open reading frames encoding functional CEST genes from *B. pumilus* and *B. licheniformis* have been described previously [15] while the gene encoding the putative CEST gene from *G. kaustophilus* has been revealed as part of the whole genome sequencing of the organism [20]. The PCR techniques used to amplify the ORFs were successful (~1.5 kb was amplified) and nucleotide sequences were verified by sequencing (data not shown).

The gene encoding the CEST from *G. kaustophilus* was also subjected to optimization using GeneOptimizer™ (Geneart GmbH, Regensburg). This included codon usage adaptation for optimal for expression in *E. coli*, which was also a success (data not shown). These 3 genes encoded proteins of 489, 484 and 499 amino acid residues, respectively corresponding to lengths.

B. Over-Expression Of Carboxyl Esterases And Purification

The pMBE95, pMBE96, and pMBE179 are pET28a derived expression vectors respectively carrying *B. pumilus*, *B. licheniformis*, *G. kaustophilus* and synthetic codon optimized *G. kaustophilus* CEST structural genes. The constructs placed the CEST ORFs in frame for expression under the T7 promoter of the pET28a expression vector and in-frame with the vector's sequence coding for 6X histidine residues. *E. coli* JM 109 (DE3) was used as the expression host and the transformed cells were grown to nearly stationary phase followed by induction of gene expression using IPTG. Fractionation studies revealed significant esterase activity in both the extracellular and intracellular soluble fractions (Supplementary Table II). Based on these observations, cultures expressing recombinant esterases were freeze-dried in order to retain the total activities from both fractions. The CEST activities from *E. coli* DE3 strains pMBE95, pMBE96, and pMBE179 were denoted as Est95, Est96 and Est179, respectively indicating CESTs from *B. pumilus*, *B. licheniformis* and *G. kaustophilus*.

The lyophilized CEST fractions were purified to near homogeneity in one step purification using HisPrep™ 16/10 IMAC Ni-Sepharose 6 Fast Flow column (Fig. 1). The molecular size of the purified CESTs was found to be about 54 kDa on SDS-PAGE (Fig. 1), in agreement with the estimated molecular mass from the translated nucleotide sequences. The purified CESTs had the following specific activities against ρ-nitrophenyl acetate; Est95 = 12.7 U.mg^{-1}, Est96 = 35.4 U.mg^{-1} and Est179 = 49.4 U.mg^{-1}) (Supplementary Table II).

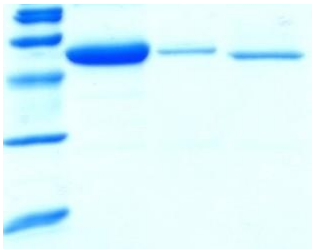

Fig. 1 SDS-PAGE analyses of Est95 (lanes 2), Est96 (lanes 3) and Est179 (lanes 4).

C. Biochemical Characterisation Of Recombinant Carboxyl Esterases

The activities of Est95, Est96 and Est179 were examined as a function of temperature. The Est95 and EstE96 CESTs of CEST from *B. pumilus*, *B. licheniformis* and *G. kaustophilus*, giving proteins with theoretical molecular masses of about 54 kDa. The predicted pI values of the proteins were 4.86, 5.38, and 5.7 corresponding to CEST from *B. pumilus*, *B. licheniformis* and *G. kaustophilus*, respectively. The deduced amino acid sequences showed at least 29% identity with the sequence of *A. oxydans* CEST, the reference Family VII lipolytic enzyme [4]. The conserved GESAG motif which contains the catalytic Ser residue could be identified within the sequences of the three proteins [15]. Showed optimal activities at 45 ^{o}C while Est179 had optimum activity between 45 ^{o}C and 55 ^{o}C. The thermostabilities of the three recombinant esterases were calculated as a percentage of the initial activity remaining

after 10 min incubation at a temperature range of 20 to 70 ° C (Fig. 2). Est95 and Est96 retained more than 95% of activity at temperature range between 25 - 40 °C. At more elevated temperatures (above 40 °C) more than 60% of initial activity was lost for both enzymes. However, Est179 retained significant activity (80-100%) between 25-60 °C. Significant Est179 activity (>70%) was lost at temperature above 70 °C. The purified recombinant CESTs displayed optimal catalytic activity at the following pH ranges 7-8, 7-9, 7.5-9 for Est95, Est96 and Est179 CESTs, respectively (Fig. 3).

Enzyme	Substrate	K_m (mM)	V_{max} (U.mg^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1}/mM)
Est95	Acetate, p-NP-C2	177.3± 27.9	3096±30 9.6	5356± 79.4	30.26
	Propionate, p-NP-C3	4.61±1 .30	327.3±49 .84	566±8 6.19	123.04
	Butyrate, p-NP-C4	4.24±2 .51	345.1±99 .85	35.6±1 0.27	8.39
Est96	Acetate, p-NP-C2	112.4± 90.49	2022±14 8.5	3199± 235.0	28.46
	Propionate, p-NP-C3	27.2±2 .5	176±11.8	278.5± 18.7	10.23
	Butyrate, p-NP-C4	23.0±5 .9	129±23.4	205.4± 37.1	9.10
Est179	Acetate, p-NP-C2	32.5±1 9.3	2469±11 5.1	9040± 421.6	282.5
	Propionate, p-NP-C3	22.8±5 .4	1252±22. 3	4586± 818.0	201.14
	Butyrate, p-NP-C4	4.8±0. 9	678.2±67 .50	2483± 24.7	517.28

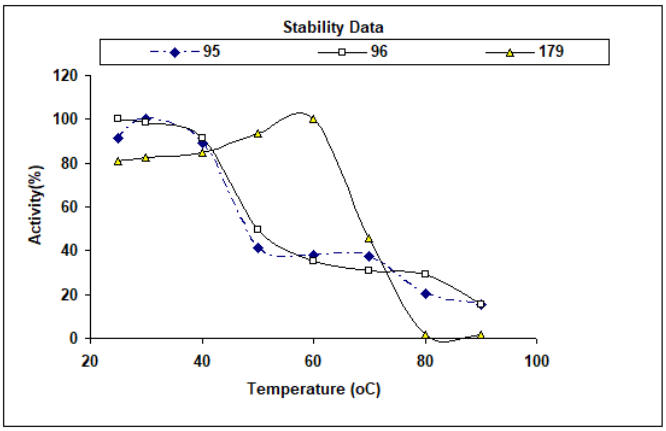

Fig. 2 Temperature stability profiles of purified Est95, Est96, and Est179 esterases. The enzymes were incubated for 10 min at various temperatures and the residual activity was measured under the standard reaction conditions.

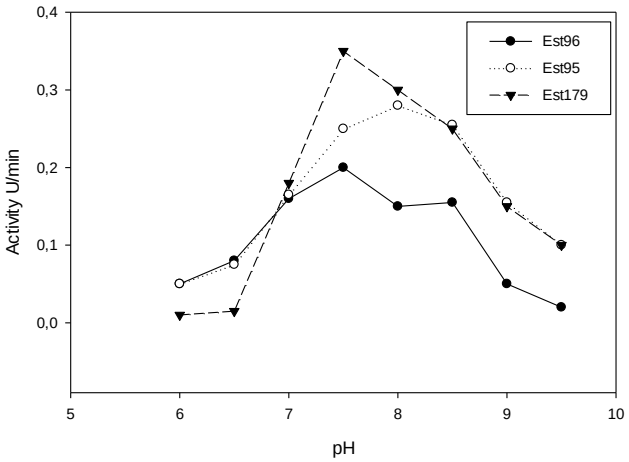

Fig. 3 pH optima profiles Est95, Est96 and Est179 esterases. The activity was assayed in buffers with different pH values.

D. Kinetic Studies

The substrate specificities of the three purified esterases against different fatty acyl chain length were determined using ρ-nitrophenyl esters of C2–C16. Substrate selectivity of the three enzymes were highest against shorter acyl chain lengths (C2-C4) (data not shown). Using three readily hydrolysed ρ-nitrophenyl ester substrates (C2, C3 and C4), kinetic constants for the three enzymes were calculated (Table I) using the Michaelis-Menten non-linear regression hyperbola. All three enzymes showed high affinity for ρNP-C4 as demonstrated by the low K_m obtained in comparison with the result obtained for pNP-C2 and pNP-C3 substrates. Specificity constants ($kcat/K_m$) ratios revealed ρNP–C3 was a preferred substrate for Est95, while ρNP–C2 and ρNP–C4 were preferred substrates for Est96 and Est179, respectively.

TABLE I
COMPARATIVE KINETIC PROPERTIES OF EST95, EST96 AND EST179

E. Far-Uv Cd And Intrinsic Fluorescence Spectroscopy

The CD spectra were measured in the wavelength range of 250 and 180 nm. The far-UV CD spectra for the three CESTs showed a double negative minimum around 210 and 220 nm with crossover positive maxima around 192 nm (Fig. 4A) which is consistent with a secondary structure consisting of α-helices and β-sheets [21, 22 and 23], a characteristic feature for α/β class proteins [24]. The intrinsic tryptophan fluorescence assay was used to assess the localized conformational changes in the tertiary structures of esterases. All esterases possess fluorophores residues (tryptophan and tyrosine residues). Therefore, tryptophan (Trp) residues were used as the reporter for local tertiary structure differences. Tyrosine and tryptophan residues were excited at 280 nm wavelength in order to detect the contribution of both residues in global conformation of the tertiary structure. All esterases had their maximum fluorescence intensities close to 350 nm (fig. 4B). Est95 and Est96 had the fluorescence intensity of 25 and 20 (arbitrary units) respectively whereas Est179 displayed the highest fluorescence intensity 38 arbitrary units.

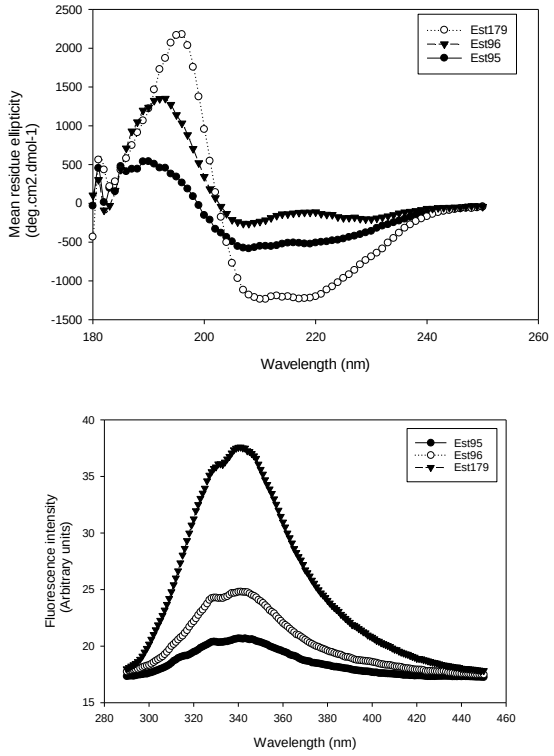

Fig. 4 Far-UV circular dichroism spectra on 5 μM of CEST from *bacillus* species (A). Fluorescence spectra on 5 μM of CEST from *bacillus species* (B).

IV. DISCUSSION

There are more than 100 gene sequences in nucleotide databases sharing 35% or more identity with the classical Family VII CEST gene derived from *Arthrobacter oxydans*. To date, only a few number of Family VII lipolytic enzymes have been expressed and characterized biochemically. It is not clear why there is a huge gap in the number of Family VII gene sequences published to date and the number of genes that have been expressed and characterized biochemically. It is significant that the catalytic activities of Family VII lipolytic proteins be elucidated as this will enable full biotechnological exploitation and understanding of structure function relationships. In this study we described the cloning, expression and characterization of three Family VII CESTs from mesophilic *Bacillus pumilus* and *B. licheniformis* species, and from a moderate thermophile, *G. kaustophilus*. Although genes encoding CEST from *Bacillus licheniformis*, *B. pumilus* and *G. kaustophilus* have been reported in nucleotide databases, their expression and biochemical characterizations has not yet been reported.

The CESTs from mesophilic *B. pumilus* and *B. licheniformis* could be readily expressed in *E. coli* DE3 cells. The expression of the CEST from *G. kaustophilus*, a moderate thermophile was achieved only after codon optimization which included the elimination of other expression-limiting factors such as internal ribosome entry sites, RNA secondary structures, and DNA repeats. A significant number of lipolytic genes are being discovered as part of whole genome and metagenome sequencings as revealed by the large number of putative lipolytic genes in nucleotide databanks. This study has demonstrated that codon adaptation of genes to that of heterologous host enables the expression of genes that would otherwise fail to express in a given heterologous host. This is more important in gene discovery studies based on functional activity screening, where structural genes remain inactive and therefore undiscovered as a result of the non-optimization of structural genes contained in genomic libraries transformed in heterologous hosts.

Production levels of 2298, 1091 and 750 Units/L could be achieved in *E. coli* DE3 cells for CESTs from *B. pumilus*, *B. licheniformis* and *G. kaustophilus*, respectively. The levels corresponded to 181, 31 and 15 mg of CESTs per litre for CESTs from *B. pumilus*, *B. licheniformis* and *G. kaustophilus*, respectively, calculated based on the specific activities obtained with ρ-nitrophenyl acetate as the substrate. A one step purification of the His-tagged recombinant CEST proteins was achieved with the use of Nickel Affinity chromatography, and this resulted in a single protein band of about 55 000 Da on SDS-PAGE gel the protein size reported for other members of Family VII lipolytic proteins [4].

The successful cloning and expression of the CESTs enabled the activity of the enzymes to be biochemically characterized. The Est95 and Est96 functioned optimally in alkaline pH values (7-9), had optimum temperatures of 45 ^{o}C and were thermolabile, losing more than 60% activity upon incubation for 10 min at temperatures above 40 ^{o}C. Similar biochemical properties were reported for Family VII CESTs derived from *B. niacini*, *Bacillus sp* Bp.7 and *Paenibacillus sp* strains [25, 26, and 27]. The biochemical properties of Est179 from *G. kaustophilus* are in accordance with the properties of est55 CEST isolated from *G. stearothemophilus* with pH optima between 8 and 9, optimum temperature range of 30-60 ^{o}C and thermostable at 60 ^{o}C [5]. Est95 and Est96 preferred ρ-NP-C3, while Est179 showed maximum activity against ρ-NP-C4. For Est95 and Est179, the results were corroborated by kinetic studies that revealed ρ-NP-C3 and ρ-NP-C4 as preferred substrates. Based on substrate profiles, Est95, Est96 and Est179 are classified as CESTs because of their preferences for short p-nitrophenyl ester substrates and lack of activity on acyl esters with n-acyl chain lengths greater than 10.

The primary structure analyses of the three CESTs reveal that the catalytic triad consists of Ser, His and Glu sharing with acetylcholine esterase a rare use of Glu instead of Asp as the active site carboxylate [28]. Protein similarity analyses of the CESTs from mesophilic *Bacillus* and *Geobacillus* reveal significant amino acid sequence identity, an indication that they derive from a common ancestral version, but differed in biochemical properties such as thermostability probably as a result of protein evolution which is crucial for organismic adaptation and fitness [29].

A comparative analysis of the structures of CESTs from thermophilic *versus* the mesophilic counterparts renders possible, the investigation of thermostabilizing factors. Far-UV CD spectra for the CESTs displayed different degrees

of ellipticity for the mesostable (Est95 and Est96) *versus* the thermostable enzyme (Est179), a possible indication of an increased number α-helices in Est179. This is further supported by previous studies that have suggested that most thermostable proteins have high contents of α-helix [30, 31, and 32].

On the other hand, the high intrinsic fluorescence intensity exhibited by Est179 indicates that Trp residues are more exposed to solvent in this thermostable CEST when compared to the mesostable counterparts. This was in agreement with the fluorescence spectrum of thermophilic esterase reported by [21]. According to [33], Trp residues may contribute to structural stability by their hydrophobicity and π-cation interactions. It is noteworthy that the 3D structure of *G. stearothermophilus* CEST, a member of Family VII lipolytic proteins is available [10]. This could facilitate protein modeling to deduce the structural basis for enhanced thermostability which is one preferred prerequisite for CEST implementation in biocatalytic processes.

V. Conclusion

CEST from mesophilic *B. pumilus* and *B. licheniformis* species and a putative CEST from *G. kaustophilus* HTA426 were successfully cloned, over-expressed in *E. coli* and purified to near homogeneity in one step purification using nickel affinity chromatography technique. The study demonstrated the significance of gene codon adaptation which enabled successful expression of the *G. kaustophilus* CEST gene in *Escherichia coli*. The adoption of gene optimization strategies could greatly facilitate the expression of genes discovered during genome and metagenome sequences that would otherwise fail to express in heterologous hosts. The CESTs were active toward short-length p-NP esters, with high stability and activity under alkaline pH. The CEST from *G. kaustophilus* is a good candidate for application in biotechnology industry given its thermostability and activity in alkaline pH. The *Geobacillus stearothermophilus* CEST is available as template for three-dimensional structure modelling to elucidate structure-function properties of Family VII lipolytic proteins allowing rational protein design and tailoring the enzyme for a given application.

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