Human carboxylesterase 2: Studies on the role of glycosylation for enzymatic activity

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Abstract

Human carboxylesterase 2 (hCES2) is a glycoprotein involved in the metabolism of drugs and several environmental xenobiotics, whose crystallization has been proved to be a challenging task. This limitation could partly be due to glycosylation heterogeneity and has delayed the disclosure of the 3D structure of hCES2 which would be of utmost relevance for the development of new substrates and inhibitors. The present work evaluated the involvement of glycans in hCES2 activity and thermo stability in an attempt to find alternative active forms of the enzyme that might be adequate for structure elucidation.

Partial or non-glycosylated forms of a secreted form of hCES2 have been obtained by three approaches: (i) enzymatic deglycosylation with peptide N-glycosidase F; (ii) incubation with the inhibitor tunicamycin; ii) site directed mutagenesis of each or both N-glycosylation sites. Deglycosylated protein did not show a detectable decrease in enzyme activity. On the other hand, deglycosylation mutants led to decreased levels of secreted hCES2 but the enzyme was still active. In agreement, incubation with the inhibitor tunicamycin led to decreased levels of secreted hCES2 but the enzyme was still active. In agreement, mutation of each and both N-glycosylation sites led to decreased levels of secreted active hCES2. However, the thermostability of the glycosylation mutants was decreased.

The results indicated that glycans are involved, to some extent in protein folding in vivo, however, removal of glycans does not abrogate the activity of secreted hCES2.

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1. Introduction

Carboxylesterases (CES) are a subset of esterases that belong to the α/β hydrolase family [35]. These enzymes catalyze the hydrolysis of esters, thioesters, and amides, freeing the respective carboxylic acid and alcohol [14]. Carboxylesterases are responsible for detoxifying xenogenous substrates, like pesticides and other environmental toxicants and are also involved in metabolizing drugs and/or activating pro-drugs [24]. In fact, after oxidative enzymes such as cytochrome (CYP) P450 enzymes and UDP-glucuronosyltransferases (UGTs), esterases are the third major class of enzymes involved in the metabolic clearance of currently administered therapeutic drugs [13,34]. Specific inhibitors for CESs have been reported, such as bis-p-nitrophenyl phosphate (BNPP) and benzyl [29,39]. Due to their potential pharmacological application, the search for individual CES specific inhibitors is, in fact, an active research field [20,28,32,40,41,5,7,8].

hCES have different tissue distribution, localization within the cell and substrate preferences [14] with some overlapping. Moreover they have different genetic variants resulting from alternative splicing, single nucleotide polymorphisms (SNPs) and multiple copy variants that have been shown to influence drug metabolism and clinical outcomes [16,23]. Human CES enzymes encompass a signal peptide of approximately 17 to 22 aminoacid residues responsible for their targeting to the endoplasmatic reticulum (ER) where they are retained due to a carboxy-terminal ER retention signal – the H-X-E-L (Histidine - X - Glutamic Acid - Leucine) consensus sequence that interacts with the KDEL (Lysine - Aspartic Acid – Glutamic Acid – Leucine) receptor present in the luminal side of the ER [26]. This is in accordance with the absence of CES from human plasma, contrary to what happens in most rodents [1,25].

Three main human carboxylesterases have been most studied...
among the five carboxylesterases listed in the Human Genome Organization database: hCES1, hCES2 and hCES3 [23]. Human CES1 has traditionally been the most studied hCES, however, increased attention has been devoted to hCES2 due to its role in the activa-
tion of prodrugs, its potential application in prodrug-activating 
gene therapies [30,38] and also its involvement in pre-systemic 
metabolism of orally administered drugs. Human CES2 is found in 
different tissues like the liver and kidney being in fact the major 
intestinal carboxylesterase [6] and it has been reported to be 
downregulated in some cancers [15] while overexpressed in others 
[35].

Contrary to hCES1, which has a substrate preference for esters 
with small alcohol group, hCES2 hydrolyzes preferentially sub-
strates with a large alcohol group and a small acyl group as is the 
case of irinotecan [11], some angiotensin receptor blockers as well 
as aspirin, having a consequent effect in their bioavailability 
[22,27].

While hCES1 is so far the only human CES form that has a fully 
known structure [2], the tertiary structure of hCES2 has been 
elusive to many scientists for more than a decade. hCES2 has two 
potential N-glycosylation sites, at Asn103 and Asn267 [26]. The 
purified native human enzyme has been shown to be sensitive to 
edoglycosidase H (Endo H), which hydrolyzes Asn-linked high 
mannose oligosaccharides [21]. On the other hand, recombinant 
hCES2 from HEK-293T cells has been efficiently deglycosylated 
with PNGase F but not Endo H, indicating that it was further 
modified at the Golgi apparatus [12]. However, taking into account 
that the efficiency of glycosylation can be affected by conforma-
tional limitations and by the acidity of “X” residue (any amino 
acid except proline) in the Asn-X-Ser/Thr sequons where N-gly-
cosylation may occur [31], the number of occupied N-glycosylation 
sites has not in fact been confirmed. Accordingly, for rat carbox-
ylesterases, the intracellular form is sensitive to Endo H whereas 
the secreted form is only sensitive to PNGase F [17].

The effect of glycosylation on hCES2 activity is not known and the 
data available on other carboxylesterases is not always con-
sensual. For example, Hydrolyase C, a prokaryotic or-
ganism, but active when expressed in the baculovirus/insect 
cell (Spodoptera frugiperda 1 – SF21) system [36,37]. In addition, the 
expression of a recombinant rabbit liver carboxylesterase in E. coli 
yielded an inactive protein which was attributed to the lack of 
N-glycosylation, whereas an active protein was obtained from 
yeast cell expression system (Pichia pastoris), capable of N-glyco-
sylation [18]. However, despite previous claims that, for full ac-
tivity of recombinant hCES1, expressed in the baculovirus-insect 
cell system, proper glycosylation (especially N-linked glycosyla-
tion) was necessary [10], difficulties in obtaining an active form of 
hCES2-10xHis from E. coli have recently been attributed to issues related 
to the protein being expressed in inclusion bodies and with low 
purity [3]. The authors have refolded the enzyme via buffer ex-
change and obtained an active form, thus questioning previous 
claims on the need of proper glycosylation for production of a 
recombinant active form. Additionally, [17] found that native 
deglycosylation of rat carboxylesterases (hydrolyases A and B), pur-
ified from rat liver microsomes, has no effect on enzyme activity.

The authors hypothesized that these data may point towards a 
higher relevance of glycosylation for protein stability and solubi-
lization and not as much for enzymatic activity of mature proteins.

The 3D structure of hCES2 has not been deciphered yet, mostly 
because its crystallization has been a challenging task. Glycopro-
teins are many times difficult to crystallize due to the hetero-
genicity of N-glycan structures and conformations at the surface of 
proteins and therefore we considered the production of non-gly-
cosylated forms of hCES2 in order to obtain alternatives for crys-
tallization and diffraction studies. However, since these new forms 

would only be useful for structural studies if they remained active, 
the main goal of the present work was to evaluate the impact of 
glycosylation in hCES2 activity. For that purpose, several strategies 
[4] were tested to prevent or remove glycosylation, and test the 
effect on enzyme activity and stability, namely: recombinant 
hCES2, secreted from HEK-293T cells [12], was enzymatically de-
glycosylated (1); N-glycosylation was inhibited during production 
(2) or the N-glycosylation sites were mutated (3).

2. Results and discussion

2.1. Effect of enzymatic deglycosylation on enzyme activity

In order to test if N-glycosylation was relevant for enzyme ac-
tivity, the supernatant of HEK293T cells transfected with pCI-neo 
hCES2-10xHis [12] was subjected to enzymatic deglycosylation 
with PNGase F under denaturing and native conditions. In both 
conditions, a downward shift in molecular weight of under 10 kDa 
was observed by Western Blot (Fig. 1A). In native conditions the 
results indicated incomplete digestion that could not be improved 
with a longer incubation period, or by increasing the amount of 
enzyme. This can be explained by limited accessibility of the en-
yyme to one of the N-glycosylation sites. Deglycosylation of pur-
ified hCES2-10xHis [12] showed a decreased level of detection 
using the glycoprotein detection method ProQ Emerald 300 with 
the deglycosylated bands only being faintly detected probably due 
to non-specific binding or to O-glycosylation (Fig. 1B).

Enzyme activity was assessed on supernatants subjected to 
native deglycosylation, (PNGase +) and compared with the control 
(PNGase-) as described in the Materials and Methods section. No 
evidence of decay in total hydrolytic activity was observed as 
specific activities were respectively 93.9 ± 3.7 pM s⁻¹ µg⁻¹
(PNGase +) and 88.9 ± 6.4 pM s⁻¹ µg⁻¹ for PNGase- and PNGase +
samples, respectively. It can therefore be concluded that the pre-
seence of N-glycans in the mature form of the protein – fully pro-
cessed through the cell machinery – is not essential for protein 
activity.

These results are in accordance with the observations of [17] 
which reffered that rat liver hydrolase A and B remained active 
after enzymatic deglycosylation.

Fig. 1. Enzymatic deglycosylation of hCES2-10xHis with PNGase F. (A) Western Blot 
demonstrating size reduction upon denaturing and native deglycosylation of su-
pernats (25 µg of total protein per well). (B) Denaturing and native deglycos-
lylation of purified hCES2-10xHis and staining with ProQ Emerald 300 (5 µg of total 
protein per well). Lanes: 1 – hCES2-10xHis under denaturing conditions (PNGase F 
–); 2 – hCES2-10xHis under denaturing conditions (PNGase F +); 3 – hCES2-10xHis 
under native conditions (PNGase F –); 4 – hCES2-10xHis under native conditions 
deglycosylated (PNGase F +).
In order to fully confirm the number of glycosylation sites, as well as the individual relevance of each site for activity, glycosylation was prevented through a different approach that consisted in using site directed mutagenesis to replace the asparagine residues in the Asn-X-Ser/Thr sequons, by glutamine thus precluding binding of the sugar moieties to those putative sites.

For that purpose, each or the two potential N-glycosylation sites were mutated resulting in three mutated forms of the protein which lacked either the first (p.Asn175Gln, Glyco1), the second (p.Asn340Gln, Glyco2) or both (p.Asn175Gln/Asn340Gln, Glyco1+2) glycosylation sites (Fig. 3).

Through Western Blot it was possible to confirm a decrease in the molecular weight of the mutant forms present in the cell culture supernatants in comparison with the positive control (fully glycosylated hCES2-10His); as expected the difference in weight was larger in the case of the double mutant (Fig. 4A) and consistent with the decrease observed upon culturing in the presence of tunicamycin. These results unequivocally confirmed that the two previously described putative glycosylation sites of hCES2 are in fact glycosylated.

Relative expression of hCES2-10His also decreased in the case of the mutants according to relative band intensities (Fig. 4A): 100%, 73%, 66% and 22% respectively for PC, Glyco2, Glyco1 and Glyco1+2. These results are in agreement with those obtained for protein expression in presence of tunicamycin. In this case it could also be due to folding impairment in the absence of the glycans that lead to protein degradation in context of the quality control mechanism associated with calnexin in the endoplasmic reticulum, or it could be due to intracellular accumulation of the protein.

The supernatants containing the mutated forms of hCES2-10His were tested for enzymatic activity (normalized to total glycosylated protein with subsequent degradation due to the quality control mechanism of glycoproteins involving calnexin in the endoplasmic reticulum [33] since we observed that tunicamycin did not affect cell viability or total protein concentration. The lower levels detected in the supernatant may also be due to intracellular accumulation of the non-glycosylated protein. Further experiments are required to clarify this issue.

Accordingly, supernatants from TM+ cultures had significantly lower hydrolytic activity towards 4-MUBA than those of the control cultures. However, taking into account that fainter bands were observed for the TM+ cultures, the observed reduction in 4-MUBA hydrolysis is more likely to be due to the lower amount of hCES2-10His expressed in the presence of tunicamycin rather than from an effective decrease in enzyme catalytic ability. Moreover, the reduction in activity is not as accentuated as the decay in band intensity. Therefore it is possible to claim that hCES2 does not fully lose its catalytic activity even when glycosylation is prevented at early stages of protein production (Fig. 2B). These results are in accordance with results from other authors that demonstrated to be possible to produce an active recombinant non-glycosylated hCES1 [3].

2.3. Mutation of N-glycosylation sites of hCES2

In order to fully confirm the number of glycosylation sites, as well as the individual relevance of each site for activity, glycosylation was prevented through a different approach that consisted in using site directed mutagenesis to replace the asparagine residues in the Asn-X-Ser/Thr sequons, by glutamine thus precluding binding of the sugar moieties to those putative sites.

For that purpose, each or the two potential N-glycosylation sites were mutated resulting in three mutated forms of the protein which lacked either the first (p.Asn175Gln, Glyco1), the second (p.Asn340Gln, Glyco2) or both (p.Asn175Gln/Asn340Gln, Glyco1+2) glycosylation sites (Fig. 3).

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The supernatants containing the mutated forms of hCES2-10His were tested for enzymatic activity (normalized to total...
protein present. Wild type hCES2-10xHis demonstrated the highest specific activity toward 4-MUBA, while Glyco 1 and Glyco 2 retained approximately 60% of the control activity and Glyco 1 + 2 dropped to 25% of activity (Fig. 4B).

These results thus indicate that the loss in the relative activity of the supernatants is more likely due to lower expression of the mutants than to loss of catalytic ability of the protein upon loss of glycosylation. This conclusion contradicts the observation of other authors who have not been able to obtain active carboxylesterases (rat and rabbit) upon expression in prokaryotic systems [18,36,37] but are in agreement with recent studies demonstrating that it is possible to obtain active hCES-1 from E. coli upon post purification refolding, despite the lack of glycosylation machinery in prokaryotes [3].

2.4. Thermal stability of N-glycosylation mutants

As previously referred, the tridimensional structure of hCES2 has not yet been determined. According to our experience this task has not been achieved due to difficulties in obtaining crystals of adequate size and stability for allowing diffraction studies (results not shown) which may be related to enzyme stability. Considering that conformation of the protein, its stability and crystallizing aptitude may be influenced by the glycosylation profile, thermal stability of the glycosylated protein and of the mutants was evaluated by quantifying the activity of the supernatants subjected to a temperature gradient (Fig. 5).

Stability of the mutants subjected to higher temperatures, in comparison with the respective control (activity observed at 37 °C), was not significantly affected at 41.2 °C. However, higher temperatures promoted a relative decrease in activity that was more accentuated in the case of the double mutant.

3. Conclusions

In conclusion, N-glycosylation of hCES2 is relevant for thermostability of the enzyme and also for its in vivo folding or secretion, but is not crucial for enzyme activity. Since the partial or non-glycosylated mutants are still secreted to some extent in an active form they constitute promising candidates for future structural studies.

4. Materials and methods

4.1. Cell culture and transfection

Suspension adapted Human embryonic kidney cells (HEK-293T; ATCC CRL-11268) were cultured using Frestyle 293 medium (Gibco, Life Technologies; USA). The cells were sub-cultured twice a week using an inoculum of 0.3 × 10⁶ cells/mL and incubated in a humidified atmosphere of 8% CO₂ at 37 °C, with orbital agitation at 130 rpm.

To determine cell viability and concentration the Trypan Blue exclusion method was used; the cellular suspension samples were diluted in 0.1% Trypan Blue (Gibco, Life Technologies; USA) solution prepared in PBS (Gibco, Life Technologies; USA) and the cells were counted twice on a Fuchs-Rosenthal haemacytometer (Marienfeld; Lauda-Königshofen, Germany).

The pCIneo hCES2-10xHis mammalian expression vector was generated as previously described [12]. Briefly, human recombinant CES2 gene synthetically synthesized with a C-terminal 10xHis tag (GeneArt; Regensburg, Germany) was cloned in pC1-neo plasmid (Promega; Madison, USA) using SalI and NotI restriction endonucleases (New England Biolabs; Ipswich, USA). For the formation of a full transcript containing the C-terminal histidine tag,
This page contains text about molecular biology and enzymatic activity assays. It describes the preparation and analysis of proteins, including SDS-PAGE, Western Blotting, and enzymatic activity assays. The text also includes specific procedures for deglycosylation, transfection of HEK-293T cells, and the evaluation of enzyme activity. The page includes references to various biological processes, such as cell culture and protein purification. The text is dense with scientific terminology and data, typical of a research paper. It also includes tables with primer sequences for mutagenesis.
in the reaction mixture never exceeded 1% (v/v) in order not to affect hCES catalytic activity [33].

4.7. Thermal stability

Supernatant samples were subjected to a non-linear temperature gradient using a BioRad IQ Cycler IQ5 (Bio-Rad, USA) for 3 min. After incubation the samples were centrifuged at 13,000 rpm, 4 °C for 15 min. The pellets, which contained denatured protein, were discarded, and the supernatants were tested for enzymatic activity as described above.

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