Pakistan J. Zool., vol. 55(3), pp 1207-1214, 2023 **DOI**: https://dx.doi.org/10.17582/journal.pjz/20180824120824

Genetic Diversity and its Impact on Post Translational Modifications of PKC and bml-Beta Tubulin Homolog Proteins in Different Species and Strains of *Sordaria*

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ABSTRACT

Protein Kinase C (PKC) and tubulin homologs are present in all eukaryotes and play significant role in growth, development and cell differentiation through phosphorylation and de-phosphorylation of other proteins. In this study, we have amplified PKC and beta tubulin homolog *bml* gene from six strains and F3 and F4 generations of *Sordaria fimicola* collected from the Evolution Canyon-1. Sequenced products of 464 bp of tubulin gene and 548 bp for *PKC* gene were aligned to observe the genetic variations between the eight parental strains of *S. fimicola* and reference sequence of *S. fimicola*. Total six polymorphic sites were observed in case of tubulin gene out of which 5 sites were common among strains isolated from the s-slope of Evolution Canyon (EC). Genetic variations in four nucleotides were observed for *PKC* gene *i.e.* C (150) T; C (186) A; C (429) G and T (521) A which were common for S1, S2 and S3 strains, while point mutation C (497) G was detected only in S2 and S3 strains. Post translational modifications (PTMs) of both proteins were predicted and compared with the reference sequences of *Neurospora crassa* and *Sordaria macrospora* by using different PTMs predictor servers. Phosphorylation and glycosylation in different species of *Sordaria* as well as *N. crassa* was calculated on Serine (S), Tyrosine (Y) and Threonine (T) residues by NetPhos and YinOYang.

Article Information
Received 24 August 2018
Revised 13 March 2020
Accepted 05 May 2020
Available online 15 November 2022 (early access)
Published 16 March 2023

Authors' Contribution
AA and RA equally contributed to perform the experimental work. MS designed the research work. SN and MGS helped in manuscript write up.

Key words
Phosphorylation, Glycosylation, Modifications, Protein, Nucleotide, Diversity, Strains

INTRODUCTION

The complexity of eukaryotic cell cannot be explained only by the number of genes and proteins but by their complex degree of regulation which includes several levels and mechanisms. Thus, one of the major challenges is to understand these systems universally in order to extract the active part of the regulatory landscape from one or correlated snapshots of a cell state. One important step of this regulation is performed after translation, where protein function is defined by the interplay between protein–protein interactions (PPIs) and post-translational modifications (PTMs). These two mechanisms are interdependent since PPIs are described to be regulated by PTMs and intermediate enzymes are also subject of modification (Minguez *et al*., 2015; Beltrao *et al*., 2012; Li *et al*., 2013). PTMs are indeed an abundant and widely spread source of protein regulations (Beltrao *et al*., 2013). They are involved in a vast number of functions, from protein stabilizing factors to regulation of molecular switches (Van Roey *et al*., 2013).

Protein kinase C (PKC) is comprised of different groups of kinases that are involved in the phosphorylation of hydroxyl groups of serine and threonine. These kinases are activated with the help of Ca_2+ and di-acyl-glycerol. PKC enzymes have critical role in cell signal transduction cascades. The regulatory and catalytic domains are the two structural domains of these kinases. PKCs are present in all eukaryotes and play significant roles in growth, development and cell differentiation through phosphorylation and de-phosphorylation of other proteins (Penn *et al*., 2015).

For many years, PKCα was considered as an important upstream component of signalling pathways leading to cell union and relocation of cancer cells (Rabinovitz *et al*., 2004). Most of the substrates of PKC go through phosphorylation *in vitro*. These substrates initiate

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0030-9923/2023/0003-1207 $ 9.00/0

the association with the actin cytoskeleton and cell to cell connections (Jaken and Parker, 2000). Once the PKCα start phosphorylation, these substrates impact cytoskeletal dynamics that prop up adhesion and association with other proteins (Larsson, 2006).

Tubulin are phosphorylated at particular serine residues to make tubulin polymer and heterodimers (Abeyweera *et al*., 2009; Farhadi *et al*., 2006).

Oakley (2004) first time reported α, β and γ tubulin encoding genes in the *Aspergillus*. Each α and β tubulin are encoded by two genes *tubA, tubB* and *benA, tubC* respectively, while γ tubulin is encoded only by one gene *i.e. mipA*. All these genes perform specific functions such as, *tubA* and *benA* are involved in the formation of fungal hyphae, *tubB* is essential for completing sexual cycle while *β tubulin* gene is involved in the spindle formation during mitosis. β -tubulin protein is encoded by several genes in various organisms. Fungal β-tubulin is encoded by 1 or 2 genes, *tubb1* and *tubb2* genes which are involved in β -tubulin translocation. Presence of more than 1 genes of *β tubulin* in individuals indicates the complexity of gene expression to perform different functions during the life of cell. The participation of microtubules in multi processes indicates the stability of tubulin gene expressions. All these functions are controlled by post translational modifications (PTMs) on various amino acids like Threonine, Serine and Tyrosine residues of tubulin protein for the regulation of cellular activities (Jarmo *et al*., 2005). The aim behind this study was to explore the genetic diversity in bml beta tubulin and PKC proteins in *Sordaria fimicola* and different strains of this fungus collected from Evolution Canyon, Israel, which is important key station to study the genetic diversity among same species due to existence of harsh and mild environmental conditions on two opposite slopes of this Canyon. So far no PTMs have been calculated or predicted for these two proteins in this coprophilous fungus.

MATERIALS AND METHODS

Specimens

Different strains of *Sordaria fimicola* were used throughout this study. These strains were isolated from the soil and dung samples collected from the north and the south facing slopes (SFS and NFS) of the Evolution Canyon, Israel. All strains were sub-cultured under aseptic conditions at 18 °C on Potato Dextrose Agar (PDA) media. For more details about strains please see Arif *et al*., 2017.

Extraction of genomic DNA

Genomic DNA of 50 fungal isolates were extracted by the modified protocol of Pietro *et al*. (1995) and quantified by 1% agarose gel electrophoresis stained with ethidium bromide using 1Kb ladder DNA (Norgen) and image was captured under gel documentation system (Ugenius3-SynGene).

Real time PCR

PCR amplifications were performed for the amplifications of beta-tubulin *bml* and *PKC* genes using the Roche LightCycler®480 systems with similar PCR mixture composition and reaction conditions as described by Arif *et al*. (2017). The 384-well plate was used for the PCR analyses. 10µl PCR mix comprised of 2µl of genomic DNA (1 in 10 dilution of the g-DNA stock), 1µl of 10X PCR buffer (Bioline), 0.4µl of 50mM $MgCl_2$, 0.064µl of dNTPs, 0.25µl of the Light Cycler® 480 High Resolution Melting Master solution (Roche), 0.01µl of the IMMOLASE™ DNA polymerase (Bioline), 0.04µl of the forward and reverse primers each at 100µM, and 6.26µl of water.

The amplification was programmed as follows: initial DNA denaturation at 95 °C for 10 min, followed by 50 cycles each of 95 °C for 5 sec, 65 °C for 15 sec and 72 °C for 1 min ending with a final elongation step at 72 °C for 5 min. Fluorescence acquisition was obtained after each 72°C step. Products were heated to 95°C for 1 min, cooled to 40°C for one min and raised to 78°C for one second. As temperature increased gradually from 78°C to 95°C, fluorescence data were acquired continuously.

Bioinformatics tools for prediction of post-translational modifications

To calculate the YinOYang and glycosylation for bml beta tubulin and PKC proteins; YinOYang 1.2 server (accessible at: http://www.cbs.dtu.dk/services/YinOYang/) and NetPhos 2.0 server (accessible at http://www.cbs.dtu.dk/services/NetPhos/) was applied for the prediction of phosphorylation sites for these two proteins in *S. fimicola* and reference organisms.

RESULTS

The g-DNA of the six parental strains of *S. fimicola* and their subsequent F3 and F4 generations was subjected to the amplification of *bml* and *PKC* genes and was analysed using high resolution melt analysis for amplification by melting peaks and normalized melt curves. Sequenced products of 464 bp of tubulin gene and 548 bp for *PKC* gene were aligned to observe the genetic variations between the eight parental strains of *S. fimicola* and reference sequence of *S. fimicola*. Total six polymorphic sites were observed in case of tubulin gene out of which 5 sites were common among strains isolated from the s-slope of Evolution Canyon (EC) while polymorphism on 448 nucleotide position A (T) was present only in strains isolated from the n-slope of EC (Fig. 1).Genetic variations

on four nucleotides were observed for *PKC* gene *i.e.* C (150) T; C (186) A; C (429) G and T (521) A which were common for S1, S2 and S3 strains while point mutation on C (497) G were detected only in S2 and S3 strains (Fig. 2). Changes on similar nucleotides for both genes were also found in F3 and F4 generations of all the strains isolated from the s-slope of EC. Protein sequences of these two proteins were aligned with four species of *Sordaria i.e. S. fimicola*, *S. macrospora*, *S. tomanto-alba*, *S. brevicollis* and *Neurospora crassa* (Figs. 3 and 4). All the protein sequences were drawn from NCBI data base under the following accession numbers (FR774322; FR774490; FR774339; FR774529; FR774338; FR774528; FR774329; FR774532; FR774340 and FR774530). It is evident from Figure 3 that bml protein is highly conserved in all the species of *Sordaria* and *N. crassa,* while few varied regions are found among all the studied species in case of PKC protein (Fig. 4).

DISCUSSION

The current study is focused on calculating genetic variations for *bml* and *PKC* genes in six strains of *S. fimicola* and F3 and F4 generations of each strains raised by single spore isolation method, and to check the hypothesis that more variations are expected to occur in the environment that is harsh and stressed as compared to the natural environment (Saleem *et al*., 2001; Mobeen *et al*., 2022). These strains were collected from two contrasting environments and slopes (the s-slope and the n-slope) of Evolution Canyon (EC) of Israel. Sequence analyses of these two genes revealed the presence of polymorphism on various positions *i.e.* five polymorphic regions were observed in S1, S2 and S3 strains of *S. fimicola* when compared with the reference sequence of *bml* gene. These mutated sites were not found in the strains that were taken from the n-slope and natural or benign environment of EC. The parental strains of the n-slope show variations at one site that was not present in the strains of the s-slope (Fig. 1). In these strains A (T) polymorphism was found on 448 position of nucleotide. Nucleotide variations on five different positions were also observed in the S1, S2 and S3 strains that were absent in N5, N6 and N7 strains for *PKC* genes (Fig. 2). These findings are in accordance with the hypothesis that more variations are present in the strains that were isolated from the south slope of EC.

Many workers worked on the strains that were used in this study and their results sustain the current findings. Saleem *et al*. (2001) worked on wild strains of *S. fimicola* to determine the variations in crossing over and frequency of occurrence of gene conversion in these strains from EC and observed constant differences in crossing over and gene conversion between parental strains of the south facing slope of EC. They rose the F1 and F2 generations of these strains by self-cross of each strain while we raised the F3 and F4 generations by single spore isolation techniques. Our findings are supported by their work that considerable variations are found in the strains of the s-slope and their succeeding generations as well. These natural variations that passed from generation to generation could offer a source of recombination and genetic diversity by natural selection in each atmosphere.

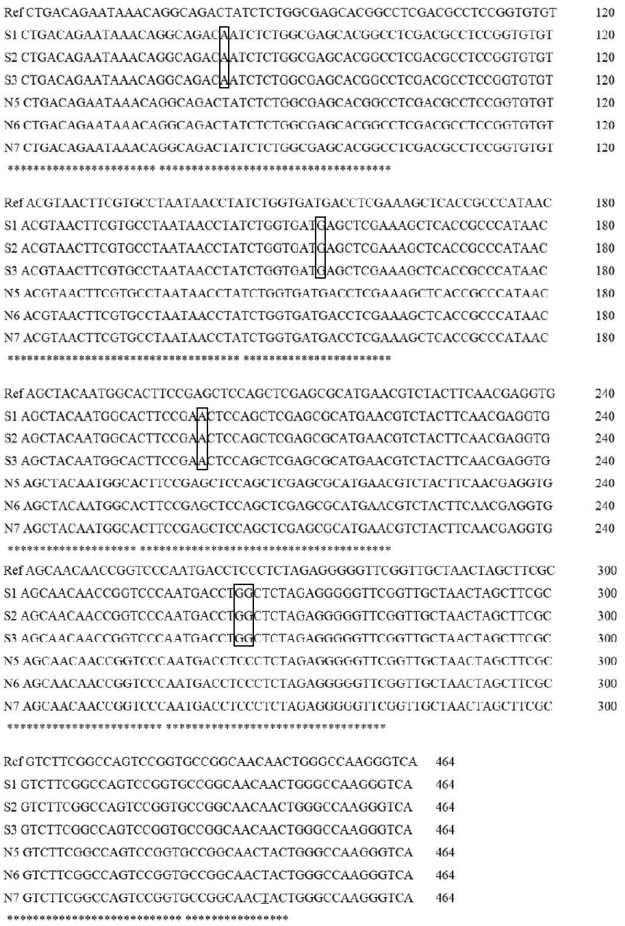

Fig. 1. Multiple sequence alignment of different strains of *S. fimicola* with reference sequence of *S. fimicola* to observe genetic variations for tubulin gene. Highlighted area indicating polymorphic sites while (*) symbol indicating conserved regions.

According to Lamb *et al*. (1998) strains that belonged to the south slope of EC had high rate of natural mutations and induced mutations compared with strains from the fresh and mild, North Slope of EC. Communal total transformation frequencies for many loci such as ascosporic pigmentation were more for three strains (S1, S2 and S3) from the s-slope, and low for three strains (N5, N6, N7)

from the n-slope. These observations further validate the present result that more polymorphic sites are present in S1, S2 and S3 strains for bml and PKC loci as compared to the N5, N6 and N7 strains. Few of these between-slope differentiation were transferred to two generations (F1 and F2), with usual natural mutation frequencies of 1.9% for the strains of the SFS (south facing slope) and 0.8% for NFS (north facing slope) of evolution Canyon. Similar between-slope variations were found for ascosporic germination-resistant to acriflavine, with high frequencies in strains from the SFS.

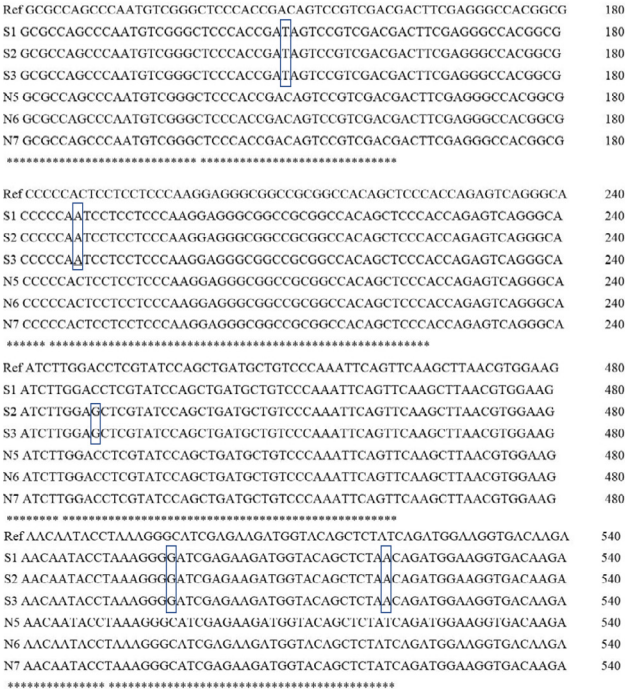

Fig. 2. Multiple sequence alignment of different strains of *S. fimicola* with reference sequence of *S. fimicola* to observe genetic variations for *PKC* gene. Highlighted area indicating polymorphic sites while (*) symbol indicating conserved regions.

Fig. 3. Multiple sequence alignment of different species of *Sordaria* and *N. crassa* to observe conserved regions of Beta tubulin homolog bml protein.

Fig. 4. Multiple sequence alignment of different species of *Sordaria* and *N. crassa* to observe conserved regions of PKC protein.

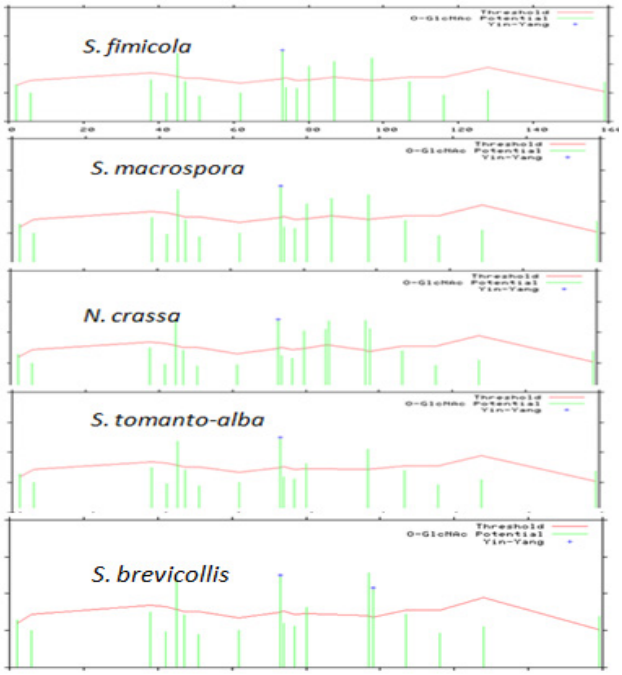

Fig. 5. Comparison of O-glycosylation and YinOYang sites in different species of *Sordaria* and *N. crassa* for BML protein.

Different strains *of Penicillium lanosum* and *Aspergillus niger* were checked under the natural environment of laboratory to observe the variations in conidial color and in other morphological characters. The rate of mutations was evidently related to whether these strains had been collected from area of high temperature and radiations or mild environment. More variations were also observed as found in the present findings that conidial color differences are more pronounced in strains that were

taken from drier, harsh, high temperature, and with much UV radiation than the opposed slope (Lamb *et al*., 1998).

Similarly, Rosenzweig and Volz (1998) studied eleven species from the n-slope and s-slope of the evolution Canyon and studied the effect of cobalt 60 irradiation on the growth rate of these species and observed the marked differences in growth rates, morphological characters and rate of sporulation in those species that were isolated from the south slope. Arif *et al*. (2017) determined the enrichment of SSR between the strains of these two slopes and found high number of short sequences repeats and point mutations in the strains of the SFS of EC. Highly polymorphic nature of V4 regions was found in the S1, S2 and S3 strains of *S. fimicola* (Arif and Saleem, 2016) while no polymorphism was found when *ITS* region of these strains were amplified by Arif *et al*. (2016). Genetic diversity for the frequency clock and mating type a1 genes were also reflected in the strains of the s-slope (Arif *et al*., 2017).

Protein kinase C (PKC) and bml homolog of beta tubulin proteins in parental strains and their F3 and F4 generations were aligned to study post translational modifications by applying different bioinformatics software. Highly conserved nature of bml protein was found between the four species of *Sordaria* and *N. crassa* (Fig. 3). Polymorphisms on 9 different residues were observed when strains under investigations were aligned with four different species of *Sordaria* and *N. crassa i.e.* mutations on 37I, 58Y, 62S, 68D, 69V, 85T, 86S, 87A, and 97T. Among these mutations; mutations at S62, D68, Y58, and 85T, S86, A87 and T97 are present only in *Neurospora crassa* as compared to the four species of *Sordaria*. *S. brevicollis* differed from other three species of *Sordaria* on two residues *i.e.* 86A and 97 (Fig. 4). This indicates that PKC protein varied at genus level and can be used to determine phylogenetic relationship between different species. Point mutations in conserved region of homologous PKC protein were identified by different workers (Detlef *et al*., 1999; Hietakangas *et al*., 2006; Gregoire *et al*., 2006; Park *et al*., 2011).

This study reports the phosphorylation of bml proteins on 5 residues of four species of *Sordaria* on similar positions *i.e.* 16S, 6T, 22T, 33Y and 42Y but in *N. crassa* the phosphorylation on similar residues were observed but at different positions (Table I). Present results revealed that glycosylation and phosphorylation on the serine/threonine/tyrosine in the *N. crassa* and *S. fimicola* showed conserved nature and these poly-modifications are homologous in all strains and controlled by similar kinases. Currently, 8 serine/threonine protein kinase genes are predicted in *N. crassa* that are involved in the phosphorylation on PKC protein while in four species of *Sordaria,* total 13 kinases are involved out of which four kinases are considered to play the important role for the regulation of phosphorylation for bml beta-tubulin protein in these species (Cdc2, CKII, INSR and Unsp). All these kinases performed phosphorylation on similar positions in *Sordaria* species but in *N. crassa* they worked on different positions (Table I).

In the same way as mentioned above Park *et al*. (2011) predicted 107 ser/thr protein kinase genes in *N. crassa* of which 89 ser/thr kinases were selected for further analysis. They studied 89 different genes during their investigation, and found that six genes are unique to filamentous fungi. Based upon the catalytic activity, PKs (protein kinases) are divided into many groups *e.g.* the AGC group includes three kinds of kinases, PKA, PKG and PKC; ARK group, the ribosomal S6 kinase, NDR (Marcote *et al*., 1992; Dickman and Yarden, 1999; Franchi *et al*., 2005). The CK1 is a small but important group in eukaryotes having CK1 protein family; the CAMK group has CAMK1 and CAMK2 protein kinases. As it is clear from the Table I that AGC group also play essential role for the regulation of PKC protein in two filamentous fungi *i.e. N. crassa* and *Sordaria* species.

It was shown that Cdc2 Kinase performed the function of phosphorylation of bml beta tubulin homolog protein on Ser16. Cdc2 activation takes place during cell cycle by binding with a regulatory subunit known as cyclin (Janke and ChloeBulinski, 2011). Tyrosination involves the joining of tyrosine amino acid on α tubulin C-terminal on the place of glutamate residue, the enzyme involved is the tubulin tyrosine ligase (TTL), this happens in the stable hetero-dimer of microtubules. Tyrosine kinase is responsible for the phosphorylation of two residual locations at Ty-33 and Ty-42 (Table I). The threonine phosphorylation takes place on two positions Th-22 and Th-6 with the involvement of CkII and Unsp protein. Casein is the acidic protein phosphorylated by CkII enzymes. Phosphorylation of th-22 is unspecified. β tubulin is a basic unit for the construction of all microtubules, but on the basis of PTMs microtubules vary. These structural modifications have been conserved during evolution and specify them in their functions (Jennetta *et al*., 2008). The assemblage of microtubules makes cytoskeleton and α/β heterodimers which are responsible for the formation of various structures like spindle fibers during mitosis. PTMs like acetylation, phosphorylation and Glycosylation in the tubulin protein leads the particular functions and also maintains their stability. Post translational modifications are a reversible process which takes part in the cell development, growth and transport etc (Ahmed *et al*., 2007).

S. fimicola, S. brevicollis, S. macrospora, S. tomanto-

alba, and *Neurospora crassa* revealed predicted PTMs on similar residues of tyrosine, threonine and serine. But there are some variations in PTMs of *N. crassa* at same gene which define variable nature of PTMs (Robles-Flore *et al*., 2008). By using western blot analysis activation of the PKC can be studied. The analysis showed that all expressed PKC isozymes are changed due to phosphorylation and nitration of PKC enzyme on tyrosine residue (Cristina *et al*., 2010).

Table I. Phosphorylation and O-glycosylation predicted residue sites of β-tubulin, BML and PKC proteins with different kinds of kinases involved.

Organism	Residue	Phosphorylation	Glycosylation and YinOYang	Protein kinases			
Bml protein				**Cdc2**	**CkII**	**INSR**	**Unsp**
S.f	Sr	16	-	16	-	-	-
	Tr	6,22	6	-	22	-	6
	Y	33,42	-	-	-	33	33, 42
S.m	Sr	16	-	16	-	-	-
	Tr	6,22	6	-	22	-	6
	Y	33,42	-	-	-	33	33, 42
S.t	Sr	16	-	16	-	-	-
	Tr	6,22	6	-	22	-	6
	Y	33,42	-	-	-	33	33, 42
S.b	Sr	16	-	16	-	-	-
	Tr	6,22	6	-	22	-	6
	Y	33,42	-	-	-	33	33, 42
N.c	Sr	12	-	12	-	-	-
	Tr	2,18	2	-	18	-	-
	Y	29,38	-	-	-	29	29, 38
PKC protein				**PKC**	**PKA**	**Cdc2**	**Unsp**
S.f	Sr	38,42,47,51,73,77,80,128	73	47, 128	57, 73	73, 80, 128	38, 42, 47, 51, 73. 128
	Tr	6,62,116	-	6	-	116	62, 116
	Y	106. 140	-	-	-	-	106, 140
S.m	Sr	38,42,47,52,73,77,80,128.	73	47, 128	52, 73	73, 80, 128	38, 42, 47, 52, 73. 128
	Tr	6,62,116	-	6	-	116	62, 116
	Y	106. 140	-	-	-	-	106, 140
S.t	Sr	38,42,47,51,73,77,80,128.	73	47, 128	51, 73	73, 80, 128	38, 42, 47, 51, 73. 128
	Tr	6,62,116	-	6	-	116	62, 116
	Y	106. 140	-	-	-	-	106, 140
S.b	Sr	38,42,47,51,73,77,88,98,128	73, 98	47, 128	51. 73	73, 88, 98, 128	38, 42, 47, 51, 73. 98, 128
	Tr	6,62,116	-	6	-	116	62, 116
	Y	106. 140	-	-	-	-	106, 140
N.c	Sr	38,42,47,51,62,73,80,128	73	47, 73,128	51, 73	80, 128	38, 42, 47, 51, 62, 73, 128
	Tr	6,86,98,116	-	6	-	98	73, 128
	Y	58,106,140	-	-	-	-	116

CONCLUSION

It is concluded that O-GlcNAc of protein residues threonine and serine play an essential role in the modification and regulation of the PKC. PKC stimulation

enhanced the proteins PTMs and may decrease the N-Glycosylation, although activation of PKC increases the O-linked beta-N-acetylation and tyrosine modification.

Statement of conflict of interest

The authors have declared no conflict of interest.

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