Mass Spectrometry based Identification and Characterization of 28 kDa protein from Teliospores of Karnal Bunt Pathogen Tilletia Indica

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Research

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Abstract

Background

Karnal bunt (KB) of wheat incited by *Tilletia indica* is an economically important quarantined fungal disease that cause huge economical loss to agricultural productivity.

Methods

In the present investigation, we have characterized the unique immunoreactive determinant present on the teliospore's wall of *Tilletia indica* employing proteomic and *in-silico* approaches. The 28 kDa protein from the teliospore's walls was eluted from the preparative gels and further subjected to two-dimensional gel electrophoresis. Mass spectrometry was carried out in search of teliosporic protein(s).

Results

Proteomic analysis identified and characterized proteins as small Heat shock protein (sHSP20) and Trehalose-6-phosphatase synthase (TPS) in T.indica. *In-silico* analysis confirmed the function of the identified protein as a sHSP and TPS belonging to stress response and carbohydrate metabolism. These results were further complemented to identify the homologs of proteins by sequence and structure based functional annotation using genome sequence of *T. indica.* The potential diagnostic protein present in immunoreactive 28 kDa of teliospores wall entities are sHSP and TPS.

Conclusion

This study is the first to perform finding indicated that proteins sHSP and TPS act as functional protein, involve in the pathogen protection under stress condition. Together these proteins increase our understanding of the transition from vegetative to sporulation process of *T. indica* by activation of sHSP-TPS, especially in terms of energy metabolism and stimulus responses. Identification of such protein(s) may also provide an opportunity to generate specific immunoprobes for development of rapid immuno-diagnostic assay.

Background

Tilletiaindica (syn *Neovossia indica*), a type of smut fungus is known to be causative agent of Karnal bunt (KB) disease in wheat. Being a seed borne disease, it affects the seed endosperm (Bashyal et al. 2020). Teliospores viability in soil was found at least for 3 years or extended up to longer time under favorable conditions (Krishna and Singh 1983, Zhang et al. 1984). KB disease in wheat was found as a new dreaded disease in Karnal city of northern India in 1931 (Mitra 1931) which continued to spread across all the major wheat producing regions within India (Haryana, Punjab, Himachal Pradesh, Uttar Pradesh and Tarai regions of Uttarakhand). Besides India and Pakistan, the disease has been also prevalent in several Asian countries including Afghanistan, Iraq and Iran, Nepal, South Africa, Mexico, Syria and USA (Singh et al. 2015). Wheat export to other countries has been highly restricted from KB affected regions as the disease lead to both quantitative (0.2-0.5%) and qualitative losses. Under such circumstances, wheat export is being constrained because most countries regulate the KB pathogen as a quarantine pathogen. Wheat is also economically important grain for global trading, but due to diseased wheat it has barriers on the free trade for infected teliospores of *T. indica*. The effect of *T. indica* can be circumvented by quarantining the pathogen to maintain grain quality. Export of wheat is not simple because the quarantine authorities of importing

countries are very serious to trace Teliospores of *T. indica* in wheat. Hence, it is utmost importance for the wheat exporting countries to comply the zero-tolerance limit of wheat from KB regions (Singh et al. 2005).

The current international diagnostic protocol in practice relies on tentative identification of teliopsores based on its morphology (Wright et al. 2003). In 2004, Australia has failed to detect teliospores of *T. indica* even after using the national diagnostic protocol (Tan et al. 2010). Diagnostic protocol like molecular diagnostic (Bonde et al, 1997; Frederick et al., 2000), size selective sieving techniques (Peterson et al. 2000) have been developed for detection of pathogen but these procedures are slow and labor-intensive. European countries based on USDA protocol developed their own protocol which relies on teliospores extraction and its morphological identification at molecular level (Inman *et al*. 2003; Anonymous 2004). Though these all protocols based on morphology can be confused with other *Tilletia* closely related species like *T. horrida, T.barclayana , T.walkeri* that infect rice, *Panicum* spp and Ryegrass, respectively (Chesmore et al. 2003). In earlier studies, diagnostic methods like enzyme linked immuno sorbent assay (ELISA), seed immunoblot binding assay (SIBA), dyed latex bead agglutination test, immunofluorescence staining test (IFST), immuno-dipstick assay and lateral flow immuno-assays (LFID) involved the use of anti-teliospore antibody (Kumar et al. 1998., Gupta et al. 2000., Kesari et al. 2003., Singh et al 2011).

Research performed in the previous years has resulted in the development of diagnostic methods such as SPR using anti-mycelial and anti-teliospore antibodies for diagnosis of KB disease (Gupta et al. 2001., Singh et al. 2011.) However, the major obstacle in detection of KB teliospores is the cross reactivity of anti-teliospore antibodies with other bunt and related fungal pathogens. Hence, there is a need of characterization of unique immunoreactive antigen that will enable to develop specific immunoprobe for detection of teliosporic protein of *T. indica* for specific and differential diagnosis. There is a considerable overlap in identifying features among the different cross-reactive species making it difficult to detect the correct species-specific pathogen. Due to these challenges in morphology identification, a number of studies have been focused on the detection for correct identification using proteomics-based methods such as 2-D MALDI-TOF with overall success. There is a considerable overlap in those measurement among the different cross-reactive species that making it difficult to detect the correct specific identified pathogen due to challenges in morphology identification (Singh et al. 2013). Various studies have focused on the detection of pathogen for the correct identification using different Proteomics and bioinformatics techniques (Vithanage et al. 2017). These are highly effective technology for studying protein expression (Jadhav et al. 2018). Advantages of Proteomics is used to discover novel proteins in poorly annotated organisms (Proffitt et al. 2017). Current fungal proteomics involved 2-D SDS PAGE and protein extraction via gel elution methods (Kwok et al. 2020) followed by mass spectrometry for the identification of protein. In this work, a new gel eluted protocol was applied to protein band extraction in order to perform the peptide fragmentation analysis/ protein identification of the fungal protein of teliospores of T. indica pathogen. Immunodiagnostic methods are most efficient and have been successfully employed exclusively for the detection and diagnosis of KB. In the previous studies from our lab, 28 kD teliosporic wall protein of *T. indica* was identified as a unique diagnostic antigen without showing any cross reactivity with other pathogens related to basidiomycetes family (Singh et al. 2013). The development of effective potential immunoprobes or biomarker requires identification and characterization of diagnostic antigen.

Keeping in view, the present study was undertaken to characterize the nature of 28 kDa protein using two-dimensional gel electrophoresis and tandem mass spectrometry (Singhal et al. 2015). Analysis through proteomics-based approaches can provide more accurate and complete information instead of genomics and transcriptomics based approaches ,because expression of proteins is not only regulated at transcriptional level but also at the translational level (Humphery-Smith et al. 1997 and Manzoni et al. 2018). Furthermore, three-dimensional structure

prediction, physiochemical and functional analysis was done through *in-silico* tools. The integrated proteomics and *in-silico* approaches were used for identification and characterization of unique teliospores's wall protein of *T. indica*.

Materials & Methodology

Source of Karnal bunt infected wheat grain

Karnal Bunt infected wheat grains were collected from (IIWBR) Indian Institute of Wheat and Barley Research, Karnal and Punjab Agriculture University (PAU), Ludhiana.

Collection of teliospores and extraction of teliosporic cell wall protein

Teliospores of *T. indica* were extracted from the contaminated wheat seeds using forceps, needle, surgical blades and brush. Seeds were incised using a needle to loosen the spore mass. Teliospores released by shaking the incised seeds were used as a source of immunogen. Teliospores were mechanically lysed by grinding in mortar pestle using in liquid nitrogen followed by adding 0.5% SDS as an extraction buffer to lyse the spores for obtaining solubilized protein. Crude lysate was centrifuged at 10,000 g for 15 min at 4 °C twice subsequently to remove particulate debris. Supernatant was centrifuged at 10,000 g for 10 min at 4 °C. and transferred to fresh Eppendorf. 2mM Phenyl Methyl Sulfonyl Fluoride (PMSF) was added to the supernatant solution and stored at -20°C for further use. Protein was estimated using bovine serum albumin (BSA) as standard according to Bradford method (Bradford 1976).

Preparative SDS PAGE

SDS-PAGE was performed to resolve proteins and polypeptides bands of range 14-90 kDa. The SDS (12%) was used to analyze solubilized teliospore's protein. Preparative gel electrophoresis was carried out using mini-PROTEAN Tetra Cell, Bio-Red. 12% acrylamide gel was cast in the 1mm internal diameter gel to a height of 7 cm with a 2.5 cm 5% stacking gel. The gel was run at 100V at room temperature and once the tracker dye reached to bottom of resolving gel. Gel was fixed in Coomassie brilliant blue for 2-3 hours after electrophoresis Target 28kDa protein band was collected through preparative SDS-PAGE (Singh et al. 2013).

Two-Dimensional Gel Electrophoresis (2-DE)

Teliosporic wall 28kD protein band was eluted from preparative gels of crude protein by gel elution protocol using our lab made two different elution buffers A and B (Patent no. 345176). Eluted bands were incubated with 1000 µl of buffer A for 5 mins and rinsed with distilled water. Excised gel pieces were crushed with the help of mortar and pestle using buffer B on ice and incubated for 1 hr at 4°C. Mixture was then centrifugation at 12,000 g for 20 min at 4°C and supernatant was precipitated using triple volume of chilled TCA/Acetone (10% w/v) overnight. Precipitated protein was recovered by centrifugation at 10,000 g for 20 min at 4°C. Pellet was washed with chilled acetone and centrifuged at 10,000g for 10 min at 4°C. The residual acetone was removed by air drying. Precipitated protein was further dissolved in 25 µl of rehydration buffer (2%[w/v] CHAPS, 2M thiourea, 8M Urea, 50 mM DTT) and stored at -20 °C.

Protein Quantification:

Protein Concentration was estimated by Bradford's dye binding method (Bradford 1976). Different Bovine Serum Albumin (BSA) concentration was used to plot the standard curve.

Two-dimensional gel electrophoresis (2–DE)

Protein sample (125 µg) was dissolved and rehydrated in 125 µL of IEF rehydration buffer with 0.01% [WV] bromophenol blue on to IPG strip of range 3-10; for 16 h for 2-Dimensional electrophoresis according to Bio-Rad protocol. Isoelectric focusing (IEF) was performed by using Bio-Rad Protean IEF Cell system following slight modification on Fragner et al 2009: 250 V for 1 hr, 1000 V for 1 hr, 5 h at 10,000 V, 250 V for 1 hr, followed by 4 hr gradient from 1000 to 10,000 V, focused on 20000 V hr at 10, 000 V. Maximum current was kept at 50 mA. Strip was then reduced and alkylated by using equilibration buffer I (6 M urea, 0.375 M Tris (pH 8.8), 10% SDS, 20% glycerol and 130 mM DDT) and equilibration buffer II (6 M urea, 0.375 M Tris (pH 8.8), 10% SDS, 20% glycerol and 130 mM Iodoacetamide), respectively. Second-Dimension electrophoresis was performed using Mini-PROTEAN Tetra Cell (Bio-Rad) with 12% acrylamide gel at 100V until the bromophenol blue dye reached the bottom of the gel. After electrophoresis, Coomassie Brilliant Blue (CBB) G-250 was used to stain the gel for 4-6 hours and dipped the gel in solution (10% methanol, 7% glacial acetic acid (v/v) to distain thoroughly. Image of the gel was taken by alphaimager gel documentation system (Protein simple, California, USA). Spots were excised for further identification using MALDI- TOF/TOF.

Tandem Mass Spectrometry and Database Searching

In order to obtain mass spectra, in-gel digestion of proteins was done by manual excision of teliospore proteins from two-dimensional gel stained with Commissive Brilliant Blue. These excised protein spots were suspended in 10% glacial acetic acid and then subsequently destained at 40°C using 50 mM NH_4HCO_3 in 50% (V/V) methanol for 1 hour. Vacuum centrifugation is used to completely dry out gel particles for peptide digestion with 5 ng/µl of trypsin for 16 hours at 37°C. 0.1 % trifluoroacetic acid (TFA) solution in 50 % acetonitrile was used to extract digested peptide fragments and then resuspending it in 5 mg/ml of α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile containing 0.1 % TFA. Further digested peptides were subjected to tandem mass spectroscopy (ULTRAFLEX III TOF/TOF, Bruker Daltonics). Tandem Mass Spectrometry (MS/MS) spectra were acquired with 2500 laser shots per fragmentation spectrum at 1600 laser shots per spectrum. MS/MS fragmentation spectra of precursor ions were obtained from strongest ten peaks from MS spectra. Spectra analysis and peak list file formation has been done by Flex analysis software 3.0 (Bruker Daltonics). MASCOT (http://www.matrixscience.com) search engine was used to search peak list files on NCBI (http://www.ncbi.nlm.nih.gov) nonredundant database version 79353501, 20160114 sequences and 28992349963 residues for “Fungi”. Search parameters were followed to proteolytic enzyme, trypsin; variable modifications, oxidation (M); taxonomy, Fungi; max missed cleavages, fixed modifications, carbamidomethyl (C); peptide mass tolerance, 500 ppm fragment mass tolerance and molecular weight at 2 Da. A protein is said to be confident in identification if protein score is more than 70 %.

In-silico approaches

Sequenced genome of *T. indica* (Kumar et al. 2017) was used for identification of candidate’s protein. Identified proteins from *T. indica* genome were used for further sequence and structure based functional annotation. Sequence of identified proteins were retrieved from NCBI conserved domain database (CDD) and BLAST tools to identify and select sequence showing homology with the proteins that have structural and functional similarity with identified candidate proteins. InterProScan was used with different protein recognition methods from the InterProScan consortium for identification of motifs. Motifs act as signature to define a protein in protein family, such as enzyme in which motifs are involved with catalytic function (Mitra et al. 2016).

Physicochemical Properties and Functional Characterization

Physicochemical properties including the number of amino acids, molecular weight aliphatic index (Ikai et al. 1980), instability index (Guruprasad et al. 1990) theoretical isoelectric point (pI), Grand average of hydropathy (GRAVY) (Kyte et al. 1982) of proteins were assessed by ExPASy Protparam tool (http://web.expasy.org/protparam/) (Gasteiger et al. 2005).

Structure Validation and Analysis

Three-dimensional structures of identified proteins were predicted using RaptorX (http://raptorx.uchicago.edu.) and visualization done by Chimera software (Pettersen et al. 2005). Ramachandran plot and protein stability analysis and validation of each protein model was done by various bioinformatics tools like RAMPAGE server (Lovell et al. 2002), DALI server (Holm et al. 2020) and ProQ server (Wallner et al. 2003). Modeled protein structures were visualized and analyzed by PyMol software (Seelinger and De Groot 2010).

Protein-Protein Docking

Protein-Protein docking was done using ClusPro server (Comeau et al. 2004a; Comeau et al. 2004b). PIPER, a docking program with Fast Fourier Transform (FFT) correlation approach was used to calculate the docked complex energy with protein interaction (De Virgilio et al. 1994). Much fewer near native structures are only retained, because of the more accurate pairwise interaction potential of PIPER. Algorithm clusters the structures by considering pairwise RMSD as the distance measure. All bioinformatics tools and database used for sequence and structure analysis are listed below in the Table1.

Table 1: List of Bioinformatics Tools and database used for sequence and structural based function annotation.

S.No.	Web server	Function	Web address	References
1.	NCBI	Retrieval of protein sequence	https://www.ncbi.nlm.nih.gov/	–
2.	BLAST	To find similarity between sequence in the database	https://blast.ncbi.nlm.nih.gov/Blast.cgi	Altschul et al. (1990)
3.	ProtParam Tool	To evaluate physio- chem properties	https://web.ex23pasy.org/protparam/	Gasteiger et al. (2005)
4.	Functional analysis tools			
i)	Conserved domain database	To find conserved domain in the sequence	https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml	Marchler-Bauer et al (2015)
ii)	Interproscan	To categorized by predicted domain and important sites	https://www.ebi.ac.uk/interpro/	Blum et al. (2020)
5.	Structural prediction (RaptorX)	Protein structure and function prediction	http://raptorx.uchicago.edu/	Kallberg, (2012)
6.	Structural validation			
i)	RAMPAGE	To evaluate the quality of Ramachandran plot	http://mordred.bioc.cam.ac.uk/~rapper/rampage.php	Lovell, et al., (2003)
ii)	Chimera software	Visualization of modeled structure	https://www.cgl.ucsf.edu/chimera/download.html	Pettersen et al. (2005)
iii)	ProQ server	Assessment of the protein	https://proq.bioinfo.se/ProQ/ProQ.html	Wallner and Elofsson (2003)
iv)	DALI server	Comparison of protein against protein database	http://ekhidna2.biocenter.helsinki.fi/dali/	Holm. (2020)
v)	Autodock	Protein-Protein interaction	http://vina.scripps.edu/	Trott and Olson. (2010)
vi)	PyMol	Visualization of docked protein	https://pymol.org/2/	Seelinger and De Groot. (2010)

Results

To follow up on previous finding that 28kD protein was uniquely present on the cell wall of teliospore of *T.indica* that have glycosyl moiety on the cell surface (Singh et al. 2013). The unique immunoreactive determinant (pH 3-10) of *T. indica* teliosporic wall protein was obtained from the preparative gels of crude protein (Figure.1). The target protein (28 kDa) was eluted in bulk from the preparative crude teliosporic protein gel (Figure 1a) after extraction with solubilizing agent. For the proteomic analysis, eluted protein was subjected to 2-DE. 28 kD band exhibited more than one protein in the gel that was further identified by tandem mass spectrometry. This is the first study to report tandem mass spectrometry-based identification of the unique protein of teliospores' wall (Figure 2a). The protein spots identified are indicated by circles and number on the gels (Figure 2(b). Both spot 2 and spot 3 were identified as Trehalose 6-Phosphate Synthase (TPS) and the protein in spot 1 was identified as small heat shock protein (sHSP). The theoretical molecular mass of the identified protein is approximately 24kDa and 100 kDa with 6.25 pH for spot 1 and spot 2 and 3 respectively. Identity, Score, and number of matched peptides for each identified protein spot are given in Table 2.

Table 2: Identification of proteins in *T. indica* by MALDI-MS/MS and MASCOT.

Spot No.	Protein identity	Accession	Organism	Mascot Score	Peptide no	Function
1.	Small Heat Shock Protein (sHSP)	OAJ04726.1	*T. indica*	74	5	Chaperone activity
2 & 3	Trehalose-6-Phosphate Synthase (TPS)	Q9UUI7	*S. cerevisiae*	94	12	Involved in Carbohydrate Metabolism and Transportation

Retrieval of homologs of identified putative proteins from reference genome and its sequence and structure based functional annotation

Sequenced genome of *T. indica* was used for identification of proteins (Kumar et al. 2017). The sequence of candidate proteins was retrieved in FASTA format from NCBI/Swiss prot database with Accession No. OAJ04726.1 and Q9UUI7; entry name: Small Heat Shock Protein (sHSP) and Trehalose-6-Phosphate Synthase (TPS). The homologous sequences were searched in the *T. indica* Karnal isolate (TiK_1) genome sequenced in our lab. However, a number of genomes of *Tilletia indica* have been sequenced and compared. Here, we found 1 sHSP from each having >95-100% of similarity with TiK_1 sHSP in three recently reported genomes of Tilletia indica, which having good genome contiguity, having ids KAE8217224.1 (DAOMC236408), KAE8232366.1 (DAOMC236414) and KAE8258783.1 (DAOMC236416). Moreover, 3 TPS proteins from each having ~40% similarities KAE8224634.1, KAE8225727.1, KAE8219882.1 (DAOMC236408); KAE8228286.1, KAE8231899.1, KAE8230345.1 (DAOMC236414); KAE8260959.1, KAE8246542.1, KAE8259025.1 (DAOMC236416) were found and similar to TPS of TiK_1 genome.

One sHSP sequence showed 96.31 % homology with *T. indica* (TiK_1) genome. Three TPS sequences with 37.79, 36.75 and 37.50 % similarity with *T. inidca* (TiK_1) genome were also obtained. Besides this, we performed the similarity search with recently submitted three DAOM (Canada group) genome sequences. One sHSP and three TPS from each of the genome sequences had 95 to 100 % homology with sHSP protein sequence and 36 to 37%. The identified proteins are vital for pathogen survival and successful infection.

Physio-chemical properties are computed by ProtParam tool used to predict the primary structure that presented in Table 3. Result showed that protein of sHSP and TPS have 217 and 997 amino acid residues with an estimated

molecular weight of 24 kD and 100 kD respectively. The isoelectric point (pI) of sHSP and TPS were 6.25 and 5.84. The instability index of sHSP was 39.17 that classifies the protein as stable and aliphatic index was 63.46, indicating the thermostable nature of the protein. Similarly based on instability index and aliphatic index, TPS was identified as unstable but thermally stable protein. The calculated score of GRAVY is -0.776 and -0.331 for sHSP and TPS, respectively. Result of secondary structure predicted by SPOMA program are shown in Table 4. In sHSP, random coil (59.91%) was predominant followed by extended strand (21.20%). Alpha helix was predominantly found in the secondary structure of TPS.

Table 3:Physiochemical properties of identified Proteins

Peptide	Exp pI/Mol. Wt.	Theo pI/Mol. Wt.	Aliphatic index	Instability index	Gravy score
sHSP	7.25/28	6.25/24	63.46	39.17	-0.776
TPS	6.20/ 79	5.84/100	82.98	51.51	-0.331

Table 4.SOPMA program

Identified Protein	Alpha helix (%)	Random coil (%)	Extended strand (%)	Beta turn (%)
sHSP	15.21	59.91	21.20	3.69
TPS	42.90	34.64	17.69	4.77

Table 5: **Validation Report of Predicted 3-D Model of Identified Proteins.**

Sequence	Template	RMSD value with template (A°)	ProQ score		Dali server	Ramachandran plot (%)		
			LG score	Max score	(Z score)	Favored region	Allowed region	Outliner region
sHSP	3gt6A	1.3	0.983	0.135	11.7	89	8	3
TPS	5dxfA	1.09	5.307	0.401	45.8	93.7	4.5	1.9

The specific pathogenicity related protein may serve as potential biomarker or may use for production Monoclonal Antibody for diagnosis of Karnal bunt to detect Teliospores of *T. indica*. A detailed structure-based analysis of these candidate proteins is given in Table 3**.** Domain analysis of sHSP explained that it belongs to sHSP 20 superfamily that has alpha-alpha crystalline domain (ACD) and these are ATP independent domain. The ACD is 90-100 amino acid long represents the signature motif of sHSP. The RaptorX utilized protein structures with PDB I.D. 4rzkA, 3gt6A, 3wlzA, 5zs3A, 4FeiA as templates to predict the 3-D protein structure corresponding to chaperone. The predicted 3-D protein model had p-value and score of 1.09e-11 and 155 respectively. The predicted 3-D structure of protein by RaptorX is given in Fig 3a. The statistical analysis of predicted 3-D protein structure in Ramachandran plot suggested that 89 % of the residues in the favored region and 8% of the residues are in the allowed region with 3% of the residues are in the outlier region. Hence about 97% of the residues located in the favored and allowed categories. The RMSD of predicted protein structure with reference to template (PDB id- 3gt6A) was 1.30 A°. This indicates a good functional similarity. Furthermore, functional analysis by ProFunc software identified Pro14-Val143 as the structural motif. We observed a significant similarity using DALI results with sHPS 16.5 had a Z score of 11.7 (Table 5.) All these analyses strongly suggested that act as a molecular chaperone. These results explained the prediction of good

quality protein model (Figure 3a). Hence, these results suggested that predicted protein model by RaptorX was good quality model (Table 5).

Conserved Domain analysis of TPS, suggested that it belongs to glycosyl transferase 20 family and has trehalose-6-phosphate synthase domain. The result of InterProScan has also explained its similarity with that a motif present on TPS sequence, involved in glycosyl transferase activity. Protein Structure with PDB I.D. 5huvA, 6jbiA, 5dxfA, corresponding to trehalose phosphate synthase, were used as templates. The p-value of predicted protein structure was 1.0e-10 with 433 score respectively. Moreover, the predicted 3-D protein structure is given in Figure3b. Ramachandran plot was depicted the statistical analysis of the predicted protein model where 93.7% and 4.5 % of the residues are in the favoured region and allowed region while 1.9% of residues in outlier regions. Therefore around 98.2% of the residues were put into the favoured and allowed categories (Figure 4b). The RMSD of the predicted model with respect to the template (PDB code: 5dxfA) is 1.09 A° showing similar functionality (Table 5). Moreover, ProFunc predicted Glycogen Phosphorylase B and Glycosyl transferase 20 like conserved motif in TPS expressed close resemblance with Leu9-Pro237, Thr238-Ala446 and Lys447-Leu465 as structural motifs. We found the similar results with DALI server that showed a significant similarity with Trehalose phosphate synthase, Z score = 45.8 (Table 5). The results of sequence and structure based functional annotation suggested that the *in-silico* model proposed in the present investigation is valid and TPS possess glycosyl transferase activity. sHSP has the probability to be associated with TPS protein due to lower Z score value of sHSP. Such studies proved that *insilco* model proposed in the present work is valid and the model proves to be a rational template and provides a structural basis

The protein-protein docking between sHSP and TPS was conducted using ClusPro (https://cluspro.bu.edu/home.php) server. The structure TPS was considered as a receptor and structure of sHSP was considered as ligand during docking. The binding energy was calculated as -806.3 Kcal\mol among them. The docked complex structure was visualized by PyMol software (Fig 6).

Discussion

Diagnosis of disease and detection of infection causing agent are of paramount importance to protect seed crops prior to undertake appropriate measures for enhancing their quality. In fact, lack of pathogen detection and disease diagnosis can severely affect crop production and quality, resulting into trade loss. This is the first report of mass spectrometry-based identification of uniquely identified protein in teliospores wall of *T. indica*. Analysis of target protein from teliospores cell wall of *T. indica* revealed that protein associated with carbohydrate metabolism Trehalose 6-phophate synthase (TPS) and chaperone activity heat shock protein (HSP) under stresses. These proteins might be responsible for virulence and cause pathogenicity. The proteomic approaches complemented with *in-silico* analysis for identification of three spots with diagnostic significance (Kathera et al. 2017).

In Insilco analysis confirmed that HSP and TSP share sequence similarities with corresponding sequences in *T. indica* genome- TiK_1. The other two published genomes were not included to compare because TiK_1 was improved using all those genome sequences, So TiK_1 have the core gene set among all. This comparative information explained that the similarity in Tilletia indica strains and conserveness in all strains of Tilletia indica. *In silico* approach was used to obtain 3- Dimensional model of candidate proteins sHSP20 and TPS protein and validation has been carried out by physio-chemical parameters. The theoretical isoelectric point revealed that sHSP20 and TPS both are acidic in nature. Also, the predicted instability index value classifies that sHSP20 and TPS will be stable on expression used for further strengthen its potential. The aliphatic index indicated aliphatic side chains in proteins responsible for the thermostability. The high aliphatic index of protein make protein more thermostable as well as contains high number of hydrophobic amino acids. Hence, best suited for stress conditions, most likely in heat

stress. We observed that both sHSP20 and TPS having negative GRAVY score which indicated both are hydrophilic / soluble nature. E-value and P-value of predicted motifs are almost similar; hence, lower value of E and P validated that results are good. Z score of sHSP denotes that it has the probability to be associated with other protein (Laha et al. 2019). Results will be determined the biological function of the identified proteins of teliospores of *T. indica*. The motif analysis of sHSP20 was found the most of the conserved motif showing similarity with heat shock protein factor and are involved in molecular chaperone activity. Likewise, motif analysis in TPS showed similarity with glycosyl transferase that involved in transfer of Trehalose into adjacent protein (sHSP20). The twin proteins present in 28 kDa protein might play their integrated role during formation of teliospore wall proteins during transition from vegetative mycelia to teliosporogenesis. Moreover, the detailed information of secondary and tertiary structure of proteins are essential for the development of biomarker for diagnosis of disease. SOPMA program was used to predict the secondary structure of proteins and result analysis in Table 4. The high percentage of random coil indicated the flexibility of the protein that help in interaction and the precise secondary structure is a key element for prediction of 3-Dimensional structure.

The 3-Dimensional structure of the proteins showed desirable properties based on Ramachandran plot predictions. The Ramachandran plot shows that most of the residues are in the favoured and allowed regions with very few residues in the outlier region; this indicates that the quality of the overall model is satisfactory. Homologs of candidate immunoreactive proteins identified from *T. indica* genome were annotated for function using both sequence and structure - based analysis (Pandey et al. 2018a,2018b, 2019). Our result support that the proteins identified in this study signifies an important protein resource providing protein-protein interaction and assisting the refolding of denatured protein. Uniqueness of identified proteins in teliospores of *T. indica* species indicate their probable role in teliosporogenesis, teliospore germination and fungal growth under varied stress conditions.

The results of sequence and structure based functional annotation suggest that the proposed *in-silico* model is valid in present investigations. The sequence analysis of protein suggested that the sHSP and TPS both sharing sequencing similarity with *T. indica* genome possess chaperone and glycosyl transferase activity, respectively. The identified proteins have vital functions for pathogen survival and successful infection. The subsequent expression study of these proteins during different stages of fungal development and under abiotic/environmental stresses will envisage their role in fungal pathogenesis. Under such conditions, molecular chaperone maintains the integrity and stability of glycosyl transferase. The s-HSP-TPS twin protein might be successfully transfer the trehalose moieties to proteins of teliospore walls under heat stress conditions during teliosporogenesis. In the previous study, the 28 kDa protein was identified as glycoprotein on teliospore walls. (Singh et al. 2009). These were further annotated by both sequence and structure - based functional analysis and validated its activity and their role to act as molecular chaperone which is ubiquitous in nature besides transfer of glycosyl moieties on the teliospores proteins.

The present study suggested that teliosporic cell wall protein: sHSP has alpha-crystalline domain (ACD), function as molecular chaperone (Jovcevski et al. 2018) which is responsible for folding, assembly, translocation and degradation of protein in different usual cellular processes (Ikwegbue et al. 2017). This small HSP help to protect proteins from denaturation under stress conditions especially high temperature, consequently sHSP are responsible for increased heat tolerance capacity of a species responsible for survival in adverse conditions. It was reported that sHSP has expressed their function constitutively in microorganism like yeast (Hanazona et al. 2013), bacterial (Guzzo et al. 2012), and animals (Chen & Zhang 2015) and strongly induced under stress condition like heat and oxidative stress. Functional PpHsp16.4 from sHSP20 family was found to be required for recovery in *Physcomitrellapatens* under heat, salt and osmotic stresses (Ruibal et al. 2013., Muthusamy et al. 2017). In *Camellia ainensis*; CsHSP17.2 showed tolerance against heat in *E. coli* (Wang et al.2017a). sHSP20 expression was increased

the survival ratio of microbes under heat shock in *E. coli*. (Li et al. 2018). Although, sHSP acts as surface contaminates in other pathogens like *Mycobacteria* and *Schistosoma* with more chaperone activity and in *C. albican*, HSP is also present blastospore's cell wall surface (Urbana et al. 2003). sHSP20 abundance may enhance teliospore tolerance to extreme stress conditions like heat, cell wall degrading enzymes and acidic pH during invasion of pathogen to host and finally cause virulence.

Whereas trehalose 6-phopshate synthase (TPS) converted Glucose-6-phosphate (G-6-P) to trehalose followed by TPP enzyme using Trehalose-6-Phopshate (T-6-P) as an intermediated thus ascribing a signaling role to trehalose (Jang et al. 2003, Schluepmann et al. 2003). Trehalose: non-reducing disaccharide, and an essential for carbon metabolism to protect the cell wall from different environmental stress conditions such as heat shock, dehydration, starvation, osmotic pressure and oxidation (Avonce et al. 2006., Benaroudj et al. 2001; Hottiger et al. 1994; Argüelles, 2000; Tereshina, 2005; Thevelein 1996; Elbein et al. 2003, Iturriaga et al, 2009, Rangel et al. 2008). Trehalose is act as an important stress tolerance factor. In *Aspergillus fumigatus*, *Aspergillus nidulans* and *Magnaporthe grisea* accumulation of trehalose plays an essential role in formation of cell wall with transformation of vegetative to spore phase i.e to promote sporogenesis for its longevity (Al-Bader et al. 2010., Ni & Yu 2007). Hence, alteration in TPS affects the glycogen accumulation and spore formation of cell. (Foster et al. 2003., Wilson et al. 2007., De Silva-Udawatta & Cannon 2001). TPS expression of *S. cerevisiae* has been elevated to maintain native form of proteins and stabilize its cell structure in *Zea mays* L. and *Gossypium hirsutum* under drought (Kosmas et al. 2006., Liu et al. 2015.) The teliosporic cell wall protein contains outer layer of glycosylated protein (TPS) that expressed a unique affinity to protein than other cell wall proteins of cross-reactive species (Singh et al. 2013). Previous Studies have depicted information that heat shock proteins and trehalose synthesis are important factors in the stress: thermo tolerance in *Schizosaccharomyces pombe* (Ribeiro et al. 1997., Glatz et al. 2016).

Molecular Docking studies were carried out to predict that the interaction of these two proteins might be regulated each other's function and act as functional protein to prove a point that together act as a functional protein. In our study, *insilico* analysis explained that sHSP and TPS are interacting with lower (negative) binding energy indicated a stable system and thus likely to binding interaction. The model structure allows an apparent sight of a protein functional state of sHSP that could bind non-native (TPS) protein from cell protective pathway (Bagneris et al. 2009). Our work suggested that in *T. indica*, vegetative to spore transition may regulate by activity of identified proteins (sHSP-TPS). An increase in the sHSP and TPS profusion is correlated with phase transition. However, economically important fungus *T. indica* having very bounded information about change in phase transition from vegetative to black spore stage; teliospores. *T.indica* have very long spore phase affection kernels of wheat plant. Study suggesting that interaction of these two proteins may play a highly conserved role in spore identity. It has also been reported in *Puccinia Striiformis* that proteins are activated in stress response and promoted pathogenicity to cause infection in host cell (wheat plant) (Zou et al. 2017). In search for both the proteins such sHSP and TPS molecules that bind and stabilize in soluble forms provide stability of the structure even under various stress conditions and the production of HSP-TPS are often associated with different stress like heat, drought, osmotic starvation and oxidation. The strongly stated evidence that heat shock responses modulated by (CIF-1(GGS1/TPS1) gene positively and level of proteins high at trehalose used as carbon source and expression of Hsp26 reduced due to deletion of CIf-1 that associated with trehalose activity. HSP-TPS protein level was induced at the sporulation stage and remained high in mature spores. Deposition of protein in spore goes upto 20% at stationary phase (Wiemken et al. 1990). In *S. cerevisiae*, stress induced Trehalose accumulation also affects the production of HSPs which as mentioned above, known to provide stress protection. TPS 1 gene responsible for synthesis of trehalose, disruption in the gene prevents cells from synthesizing trehalose and including its heat tolerance respectively (De Virgilio et al. 1994). A study stated that disruption of TPS 1 gene in trehalose protein is also impaired in HSP synthesis (Hazell et al. 1995). These

studies explained that TPS has keen effect on the synthesis of HSP protein. Both the identified proteins of the teliospores work together to cause infection in plant cell. Our study was also supported our data with other organisms as mentioned above. Sequential activity of proteins was also validated through docking. In our study, we have found polar interaction between the TPS and HSP proteins (see Figure 6). In general, polar interaction of amino acids contribute to make a stabilizing structure/ form of protein. Hence, TPS-HSP fusion is an accurate model system. Our results suggested that trehalose and sHSP 20 were appear at the same time, explained their functions are also synergistic. Studies explained that trehalose enhances activity of sHSP20. sHSP 20 does not require ATP for release of protein substrate hence act as release factor. That could be very important in the spore formation. We also suggested that sHSP 20 has been transformed in the development sense, conversion of vegetative to teliospores of cell stage that is vegetative to spore stage, not only for trehalose production but even to need it for chaperone function.

In the previous study we have found that the protein was uniquely present on the teliospores of *T. indica* among all the cross-reactive species (Singh et al. 2013). Characterization of teliosporic cell wall specific protein 28kD has proven in the development of the functional protein involved in teliosporogenesis by activating spores that provoked maturation of spores. Multiplication of spores with increase in pathogen number is the cause of pathogenesis. Further characterization of the teliospore wall proteins, its glycosylation motifs and localization within the multiple-layered teliospore wall should prove valuable in the development of diagnostic reagents for T indica identification and discrimination from closely related smut fungi. The proteomic and *in silico* approaches for characterization of diagnostic proteins from teliospore wall of *T. indica* would provide insights to understand the features of teliospore wall protein. to determine its functional role in the process of teliospore germination, vegetative mycelial growth and teliosporogenesis which not only helps in development of immunoprobe but also determined its functional role in the process of teliospore germination, vegetative mycelial growth and teliosporogenesis of such important quarantined fungus belonging to Basidiomycetes. The outcome of present study may facilitate better understanding of the teliospore wall protein of KB.

These results explained the significance of 28 kDa protein as diagnostic candidate may utilize as potential diagnostic marker for the development of specific and sensitive immunodiagnostic assay for detection of infectious causing entity of KB.

Conclusions

To date, management of disease detection and its control by all diagnostic methods have not been successful. Methodology used for the development of specific and effective disease immune detection is indispensable to identify target which was only specific to that the disease-causing pathogen, *Tilletia indica.* However, so far specific determinant to *T. indica* pathogen have not been identified. Here, we have explored the protein spots which are uniquely present in the T. indica that may be used as biomarker for diagnosis. Our results unveiled that Identified protein involved in the transition from vegetative to sporulation process of *T. indica.* Identified protein plays an essential role to protect cell with abiotic stress tolerance including heat and oxidation. For this, an effective immuno-diagnostic method for detection of pathogen requires a unique and diagnostic immunoreactive protein. In a nutshell, eluted protein from teliospore's wall of *T. indica* may certainly be used as an ideal diagnostic agent against *T. indica* for the rapid and specific immune-detection for diagnosis of Karnal bunt.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting this article are include within the articles and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

PS and AK conceptualized and designed the study, PS performed the experiments, analyzed data, prepared the figure and tables and wrote the main manuscript text; AK and VP reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

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Figures

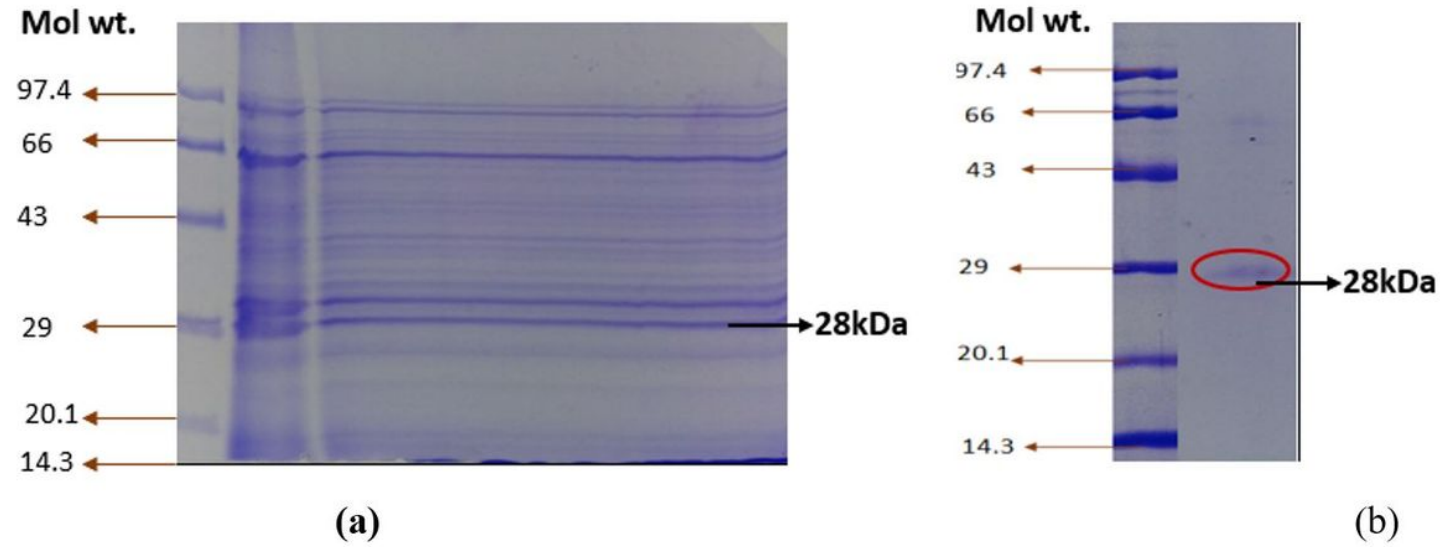

Figure 1

Preparative gel of cell wall proteins of Teliospore from T.indica. Cell wall proteins isolated from teliopsores (a) and total protein (b). Protein was eluted purified as described, 12% SDS–PAGE gel was subsequently stained with Coomassie. Eluted protein band marked with an arrow.

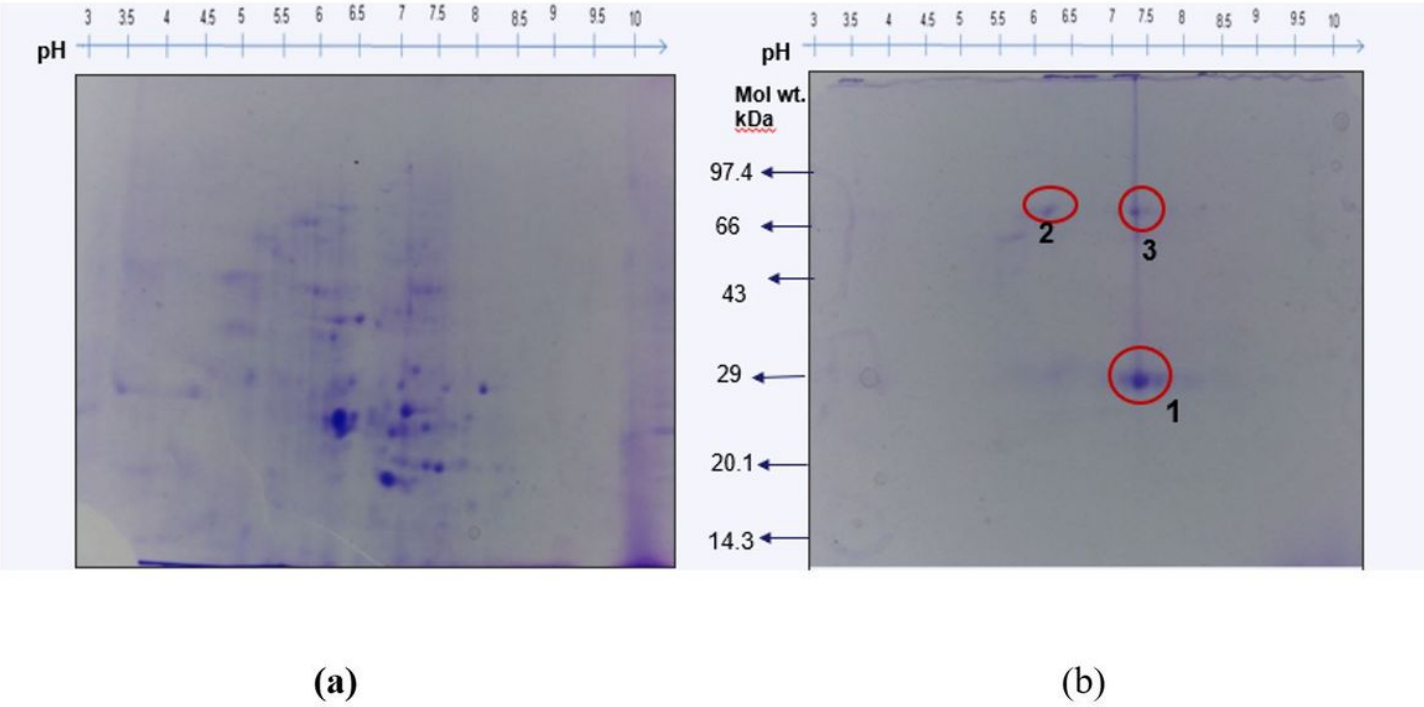

Figure 2

Two dimensional electrophoresis of Teliopsoric wall protein of T.indica (a) Total protein (crude), (b) eluted immunoreactive diagnostic protein. 2DE conditions were pH 3-10 and 12% SDS-PAGE for the first dimension and second dimension respectively. The gel was stained with Commassive. Eluted protein spot numbers refer to those given in table 2. identified by MALDI-TOF

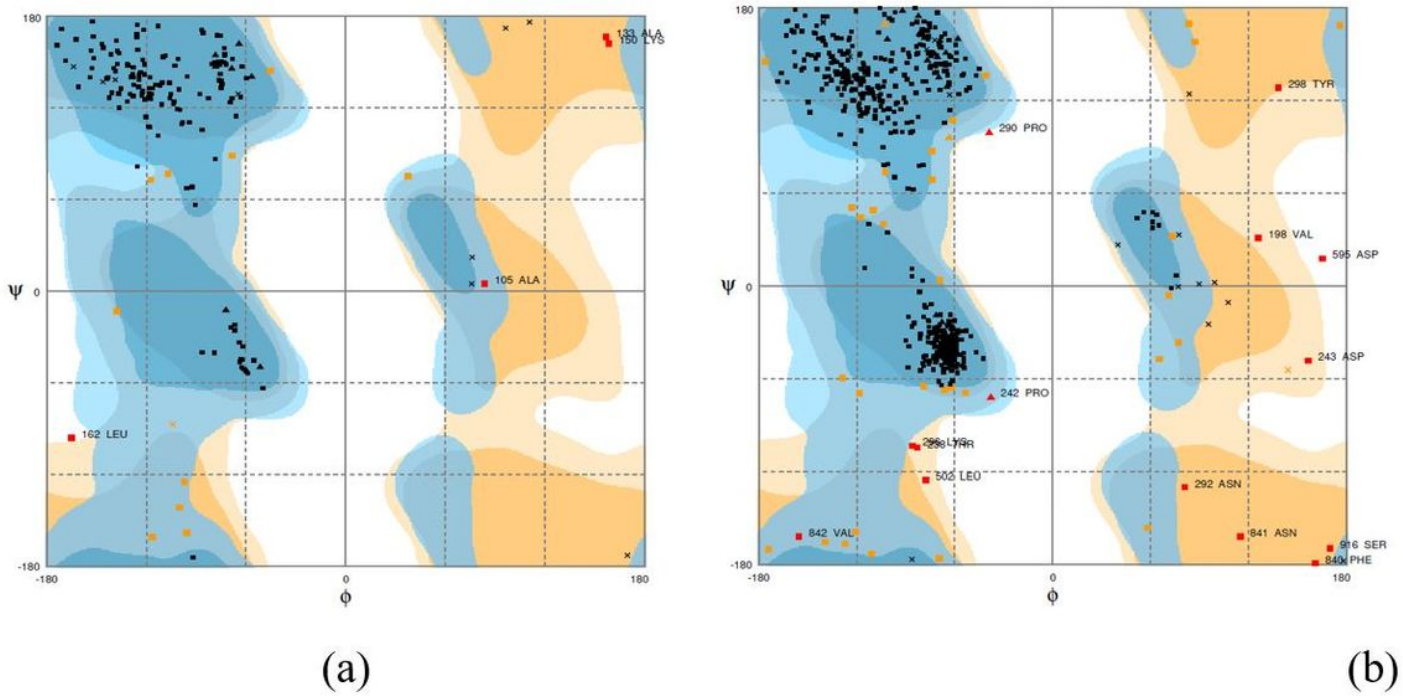

Figure 4

Ramachandran's plot of 3-Dimensional protein models predicted by RaptorX software for hypothetical and putative immunoreactive candidate. a) sHSP and b) TPS

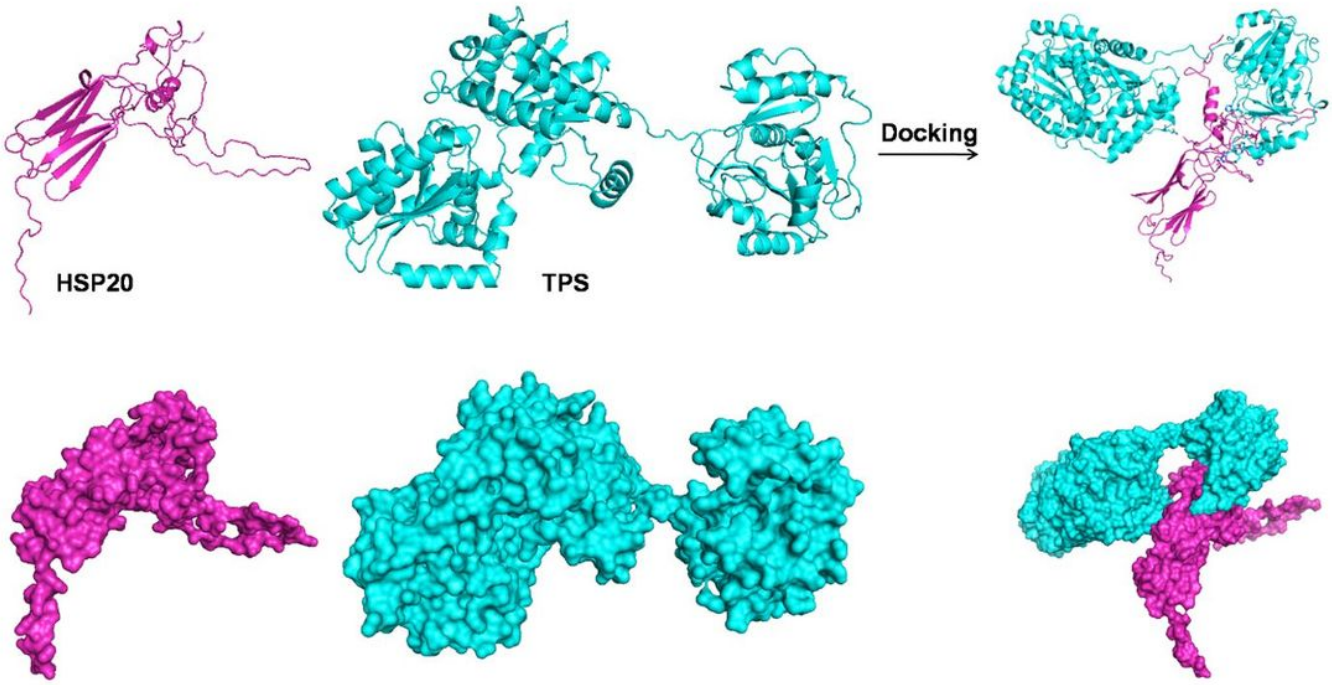

Figure 5

Protein-protein docked structure of the Heat shock protein-20 (HSP20) and Trehalose phosphate synthase (TPS) complex. HSP20 and TPS are shown in magenta and cyan respectively.