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Molecular characterization of a 42 kDa subunit pili protein of *Salmonella typhi* causes typhoid fever

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Abstract. Darmawati S, Ethica SN, Prastiyanto ME, Depamede SN, Putri EO, Kamaruddin M. 2022. Molecular characterization of a 42 kDa subunit pili protein of *Salmonella typhi* causes typhoid fever. *Biodiversitas* 23: 962-968. Blood culture is the gold standard for diagnosing typhoid fever, but it has limitations such as media and laboratory equipment, specimen volume, and examination time. However, the Academy of Pediatrics does not recommend serology due to its low sensitivity. The purpose of this study was to determine the molecular properties of the protein pili of *Salmonella typhi* (*S. typhi*) that the findings can be used to develop a typhoid fever diagnostic reagent. The SDS-PAGE method was used, as well sequence analysis with ProtParam, ProtScale, and PSIPRED. The SDS-PAGE profile reveals one major protein (42 kDa) and fourteen minor proteins. The pili protein subunit 42 kDa had an amino acid (AA) sequence with a length of 390 AA, according to bioinformatics analysis. According to the ProtParam results, the pili protein subunit 42 kDa has good stability with a value of 40 and is a hydrophilic protein with an average GRAVY value of -0.950. PSIPRED results show that among the secondary structural elements, coil strand predominates, followed by -helix and -strand. It is concluded that this protein is immunogenic and that it can be used to develop a more specific and sensitive diagnostic reagent for typhoid fever.

Keywords: Pili protein, *Salmonella typhi*, molecular characterization, typhoid fever

INTRODUCTION

Typhoid fever is an infectious condition that spreads throughout the body and is caused by the Gram-negative bacterium *Salmonella enterica* subspecies enterica serovar *typhi* (*S. typhi*) (Thieu et al. 2017; Ajibola et al. 2018). Typhoid fever is usually contracted by ingestion of water or food contaminated by fecal or urinary carriers excreting *S. Typhi*. This is one of the leading causes of mortality in many underdeveloped countries, including Indonesia. Globally, in 2010 typhoid fever was reported in 26.9 million cases (Buckle et al. 2012). World health organization estimates the incidence of typhoid fever at 21 million cases and approximately 161000 deaths (World Health Organization 2018). In Indonesia, in August 2002 and July 2004 typhoid fever is an endemic disease, with 81.7 cases per 100 000 people per year for children aged 24–60 months 148.7 per 100 000 (Wain et al. 2015). Cases of typhoid fever in the city of Semarang show that there is always an infection every month and is a disease that often occurs in large numbers. Based on the recapitulation of typhoid reports at the Semarang City Health Center, in 2015 there were 6,958 cases while in 2016 there were 7,796 cases (Andayani and Arulita 2018). Children (aged 5 to 15 years of age) are the most affected age group with a peak incidence known to occur in individuals (Pitzer et al.

2014). Due to the high incidence of typhoid in developing countries predominantly in Asia including Indonesia, prevention has become a global health priority (Jamka et al. 2019; Sahastrabuddhe and Saluja 2019).

Symptoms of typhoid infection include fever which lasts 1 to 4 weeks. Fever is accompanied by headache, chills, abdominal pain, nausea, and dry cough (Paul and Bandyopadhyay 2017). Typhoid fever commonly exhibits non-specific clinical symptoms comparable to malaria, dengue fever, influenza, leptospirosis, and Rickettsia infection, thus a definite diagnosis must be verified by laboratory tests (Azmatullah et al. 2015; Arora et al. 2019).

Blood culture is widely recommended as a method for laboratory diagnosis, however its sensitivity ranges from 40 to 80%, not all laboratories have bacterial culture facilities, it is expensive, and it takes 2-3 days to complete (Ajibola et al. 2018). Serological tests such as Widal are also often employed in laboratories because they are quick, easy, and affordable, but their sensitivity and specificity are problematic due to the frequent sharing of epitopes between the antigens of *S. typhi*, and other Gram-negative rods (Darmawati et al. 2015). It also makes use of a Rapid Diagnostic Test (RDT) for antibody detection. Typhidot, Typhidot M, Typhi Rapid IgM, IgG IgM (Combo), and Tubex TF (anti LPS antibody detection) are 50kDa outer membrane anti-proteins with a wide range of sensitivity

and specificity (47-100%) (Ajibola et al. 2018).

Pili in *S. typhi* have two basic types, namely short attachment pili and long conjugate pili. At the ends of the pili shafts are adhesive end structures that are shaped according to specific glycoprotein or glycolipid receptors on the host cell. Pili play a role in the adhesion (sticking) of bacteria to host cells, and can stimulate an immune response. Adhesion to host cells is considered a key factor during bacterial pathogenesis (Darmawati et al. 2015; Darmawati et al. 2019).

Pili are composed of pili protein which consists of several sub-units of pili protein. The pilin protein is encoded by the pilus-S gene, which is classified into the pill operon. Pili contain proteins that are able to bind to sugar molecules that make up cell membranes, called hemagglutinin proteins and proteins that can bind to host cell surface receptors called adhesin proteins (Khater et al. 2015). Hemagglutinin protein is a lectin, which can interact with carbohydrates on the surface of human erythrocytes specifically and is reversible, resulting in agglutination (clumping). Blood type A has N-Acetyl-D-Galactosamine carbohydrates, blood group B has D-Galactose, and blood group O has L-fucose (Darmawati et al. 2019).

Based on the results of an analysis of the pili protein conducted by Darmawati (2019) from isolates of *S. typhi* BA07.4 and *S. typhi* KD30.4, two main protein subunits measuring 87 and 42 kDa were observed on SDS-PAGE. Proteins that are immunogenic have a molecular weight of 10-100 kDa (Parslow et al. 2001). Based on the hemagglutination test, it was found that pilus proteins from *S. typhi* BA07.4 and *S. typhi* KD30.4 can agglutinate human erythrocytes in groups A, B, AB and O because they are able to identify receptors on the surface of erythrocytes (Darmawati et al. 2019).

Research conducted by Darmawati (2015) showed that the hemagglutinin protein of the pili sub-unit of *S. typhi* isolates at Kariadi Hospital Semarang was able to stimulate the formation of antibodies, and pili protein was an immunogenic substance and needed for the attachment of bacteria to host cells (Darmawati et al. 2015). Previous studies have looked at the SDS-PAGE profile and tested the hemagglutination of *S. typhi* BA07.4

As a result, other ways for acquiring specific antigens, such as the 42kDa protein component pili from *S. typhi*, must be explored. Pili which is made up of 14-15 protein subunits, is a tool for attachment to the surface of host cells, which is the initial step in infection, pili proteins are also immunogenic. The purpose of this study was to characterize the 42kDa subunit pili protein of *S. typhi* by examining physicochemical parameters such as AA composition, molecular weight, extinction coefficient, half-life, instability index, isoelectric point (pI), grand average of hydropathicity (GRAVY), aliphatic index, and total number of positive and negative residues, as well as secondary structure analysis.

MATERIALS AND METHODS

Culture and identification of *Salmonella typhi*

The bacteria *S. typhi* strain BA07.4 used in this study was taken from a blood culture of a positive Widal patient. API 20E and API 50CHB /E medium (Bio Merieux Inc.) were utilized for identification of bacteria (Darmawati et al. 2012). Bacterial cultures were cultured in biphasic medium (BHI Agar slant and BHI broth media) for 48 hours at 37°C without agitation (Darmawati et al. 2019). One bacterial colony on MC media was grown in 50 mL of liquid BHI as a starter, then the culture was incubated for 24 hours at 37°C with agitation. After that, 50 mL of BHI bacterial culture was added to 500 mL liquid BHI media and incubated for 6 hours at 37°C with agitation. Furthermore, the culture was implanted into biphasic media (BHI so it tilted) as much as ±50 mL and incubated for 48 hours at 37°C without agitation. The culture is ready to be harvested.

Isolation and separation of pili protein

Pili protein was isolated using the Ehara approach (Ehara 1987). After 48 hours, bacterial cultures on biphasic media were collected, and a 3% concentration of trichloroacetic acid (TCA) was added (6 mL TCA into 200 mL bacteria), held at room temperature (for 10 minutes), and centrifuged at 3000 rpm for 20 minutes at 4°C. The pellets were washed twice with 10mL of 0.1M PBS (pH 7.4) before being sliced for 3 minutes with a vortex super mixer and rested for 3 minutes before being repeated 5 times at 4°C. The resulting suspension was centrifuged for 20 minutes at 4°C at 3000 rpm, and the supernatant was protein pili.

The bacterial pili were then cut using a vortex supermixer with the step of 3 minutes the supermixer was turned on then 3 minutes the supermixer was turned off, this process was repeated 5 times with a temperature of 4°C. The results of the cutting using a vortex supermixer were centrifuged and the supernatant was taken. In the protein dialysis process, 40% ammonium sulfate was added to the supernatant (22 g ammonium sulfate in 50 mL supernatant), then the supernatant was dissolved with a stirrer at 4°C until completely dissolved. Then the supernatant was centrifuged at 3000 rpm for 20 minutes at 4°C, after which the pellet (sediment) was resuspended in 1 mL PBS pH 7.4. Pili protein suspension was put into the dialysis bag. The dialysis bag was opened by boiling in distilled water with 3 drops of 10% EDTA added for 10 minutes (Ehara et al. 1987). Dialysis of the pili protein suspension with PBS for 24 hours was carried out with the aim of removing ammonium sulfate from the pili protein suspension, the dialysis solution was replaced 2 times. The pili protein profile was analyzed using the SDS-PAGE 0.25% coomassie brilliant blue (Banta et al. 2021) stained with 0.25% Coomassie Brilliant Blue R250 after purification with 40% ammonium sulfate.

SDS-PAGE, sequencing and bioinformatics analysis of 42 kDa subunit protein

The pili subunit protein (major band) from SDS-PAGE was cut and sequenced. Protein samples were heated for 5 minutes at 100°C in a buffer solution containing 5 mM pH 6.8 Tris HCL, 2-mercapto ethanol 5%, sodium dodecyl sulfate 2.5%, glycerol 10%, with Bromophenol Blue color. Then 20 L of the sample was pipetted into the gel well. The electrodes are installed according to the poles. The voltage used is 125 mV with a running time of 90 minutes. After electrophoresis, dye with 0.05% (w/v) coomassie brilliant blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid was heated in a microwave for 15 seconds and incubated for 1 hour. The gel was rinsed by immersing it in a mixture of 30% methanol and 10% acetic acid and incubated in a water bath for up to 2-3 hours. Then to determine the molecular weight of the desired protein compared with protein markers whose molecular weight is known. The sequenced peptide segments were aligned using the basic local alignment search tool protein (BLASTP).

Physicochemical analysis of 42 kDa subunit protein

Expsy ProtParam was used to examine physicochemical data such as AA composition, molecular weight, extinction coefficient, half-life, instability index, isoelectric point (pI), grand average of hydropathicity (GRAVY), aliphatic index, and total number of positive and negative residues (<http://us.expasy.org/tools/protparam.html/>).

Protein hydrophobicity analysis

Analysis of protein hydrophilicity with the ExPASy SOSUI server (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html), and ProtScale program application (<http://web.expasy.org/protscale/>). The hydrophobicity of proteins was then determined using the HpHob/Kyte & Doolittle hydrophobic chart plot to predict solvent-accessible regions and estimate hydrophobic sites.

Protein secondary structure analysis

PSIPRED software was used to do secondary structure analysis on the ExPASy server. For forecasting secondary structure, this method is both easy and accurate. This program can estimate the secondary structure of proteins such as the beta strand, alpha helix, and coil based on the AA sequence. PSIPRED features the DMPFold tool, which may be used to see the three-dimensional structure of AA sequences.

RESULTS AND DISCUSSIONS

Profile of *S. typhi* pili protein determined using SDS-PAGE

Bacteria that have previously been discovered and cultivated are subsequently extracted pili proteins to separate pili from bacteria. Pili protein dialysis was used to concentrate protein such that pure pili protein may be produced. The findings of the pili protein isolation were subsequently subjected to SDS-PAGE in order to examine and characterize the pili protein profile, as shown in Figure 1.

The SDS-PAGE results show 15 protein bands of varying thicknesses (Figure 1). The protein concentration is indicated by the thickness of the protein band. The thicker the protein band, the higher the concentration (Sulistiyarsi et al. 2012). Among these 15 bands, there is one band of thick protein subunits called major protein subunits with a molecular weight of 42 kDa and 14 thin protein bands called minor protein subunits. Pili protein 42 kDa is an immunogenic protein based on its protein weight. Protein immunogenicity is determined by several criteria, including the protein being a macromolecule with a complex chemical structure and having foreign properties where the nature of the material is recognized as a foreign object (Chiu et al. 2019).

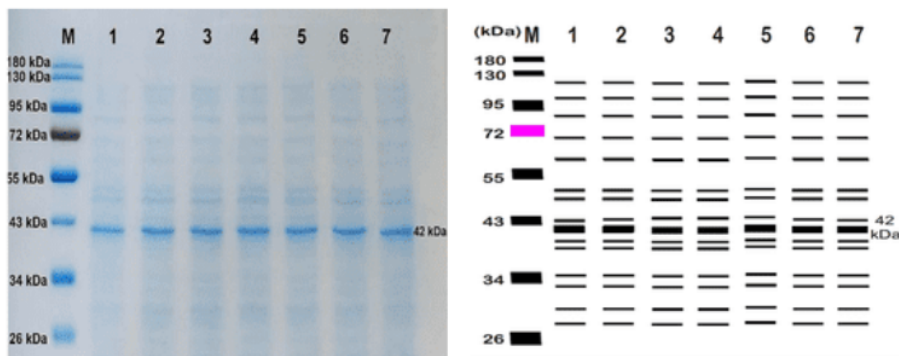


Figure 1. SDS-PAGE analysis of *Salmonella typhi* pili protein. M: Protein markers. 1-7: Pili proteins

Sequencing of *S. typhi* pili protein

Sequencing analysis was performed to determine the AA sequence in the 42 kDa protein subunit of *S. typhi*. The bands visible on SDS-PAGE (Figure 1) are cut and sequenced at Genetic Science in Tangerang, Banten, and the sequencing results are analyzed at Bio Computing House in Bogor Indonesia. To obtain homologous values and genomic information, the BLAST (Basic Local Alignment Search Tool) program was used to obtain alignment results (Figure 2).

Salmonella enterica (Accession number: EAA0576724.1) had the highest protein homolog value from the alignment results, with a percentage similarity value of 100% identity and an E-Value of 0.0, and nine other sequences were *S. enterica* bacteria with an identity similarity percentage value >99% and an E-Value of 0.0. Two proteins are said to have structural similarities in homology and folding is based on sequence similarity, if they have a percent identity value of at least 25% and an E-Value of 0.0 or 0.5 (Wong et al. 2014). The difference in nucleotide and AA homology is due to the fact that not all nucleotide changes result in a change in AA sequence (Mitra et al. 2016).

Gene information derived from the overall sequence of the 42 kDa subunit (Fig. 2) is part of the spore coat protein, CoTH, which is in the AA sequence 12,775-13,947 is a protein found in the spore coat (Figure 3).

The protein CoTH spore coat is found in the spore coat. Some adult eukaryotic and bacterial spores have a thick layer beneath their perispores called the spore coat. Álvarez-Fraga et al. (2016) discovered that spore coat proteins are involved in biofilm formation and bacterial attachment to the host in *Acinetobacter baumannii* strains (Álvarez-Fraga et al. 2016).

Composition, physicochemical and hydrophobicity analysis of 42 kDa subunit protein

The ExPASy website's ProtParam program (<http://us.expasy.org/tools/protparam.html>) was used to analyze the primary structure of the 42 kDa pili subunit protein, which included molecular weight, atomic composition, formula, atomic number, instability index, aliphatic index, and GRAVY (Tables 1 and 2). Further investigation of the ExPASy website's ProtScale program (www.expasy.org) to determine the hydrophobic level of the 42 kDa subunit protein (Figure 4).

According to Tables 1 and 2, the 42 kDa subunit pili protein of *S. typhi* has 390 amino acids with a molecular weight of 44738.20 Daltons and 20 types of AA, with lysine (L) being the dominant AA and low AA being cysteine (C). The ProtParam analysis (Table 2) revealed that the 42 kDa subunit pili protein of *S. typhi* had a value >7, which was 9.85, indicating that the protein was basic. According to Shaw et al. (2001), protein with a pI value of 7 is acidic, whereas protein with a pI value greater than 7 is alkaline. The isoelectric point (pI) is the pH of the protein's surface. The protein purification process relies heavily on pI, which is used in the development of buffers for protein purification (Wingfield 2016).

The pili protein, a 42 kDa subunit, had a stability index of 31.42. (Table 2). Protein is predicted to be stable if it has a stability index value of less than or less than 40 with a value range of 13.57 to 37.23, whereas protein is predicted to be unstable if it has a stability index value greater than 40. (Guruprasad 2019). The stability index is used to classify a protein's stability and instability (Gangadhar et al. 2016).

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MIMGVYVDKEHRVVRKRSSENGRKSFAFAHKVKNKGKNSRVQERINRKGASKEVVVKISGGAITRQGI
RNSIDYMSRESELPVMSSEGRVWTGDEILEAKEHMIDRANDPQHVMNDKGGKKNKITTQNIIVFSPVSAK
VKEPDLLESVRKTMQKYPNHRFVLGYHCDKKEHPHVHVFRIRDNDGKRADIRKDLREIRTFCEELKLG
YDVKATHKQHGHLNQSVKDAHNTAPKRQKGVYEVVDIGYDHYQNDKTKSKQHFIKLKLTLNKGVEKTY
WGA DFGDLCRSRESVKAGDLVRLKKLQKQKEVKI PALDKNGVQHGKTVHRNEWQLNLGVKGVDRTPS
ASKELV LNSPDMLLKQQQRMAQFTQQKASTLQSEQKLLKTKGKIFWGL
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Figure 2. The 42 kDa subunit pili protein sequence of *Salmonella typhi*

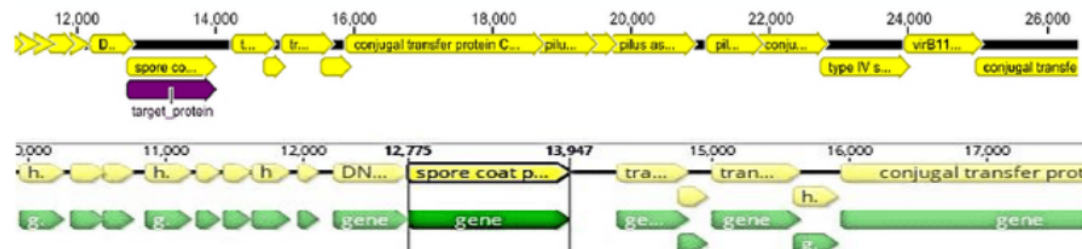


Figure 3. The 42 kDa subunit pili protein sequence of *Salmonella typhi* contains information on the location of the spore coat protein gene (CoTH)

Table 1. Amino acid composition of 42 kDa subunit protein

Amino acid composition	Percentage (%)	Content
Ala (A)	4.4	Netral
Arg (R)	6.4	Positif
Asn (N)	5.1	Netral
Asp (D)	5.9	Negatif
Cys (C)	0.8	Netral
Gln (Q)	5.6	Netral
Glu (E)	6.4	Negatif
Gly (G)	7.4	Netral
His (H)	4.1	Positif
Ile (I)	4.4	Netral
Leu (L)	6.4	Netral
Lys (K)	13.6	Positif
Met (M)	2.3	Netral
Phe (F)	2.3	Netral
Pro (P)	2.8	Netral
Ser (S)	5.9	Netral
Thr (T)	3.8	Netral
Trp (W)	1.3	Netral
Tyr (Y)	2.6	Netral
Val (V)	8.5	Netral

Table 2. Physicochemical profile of 42 kDa subunit protein

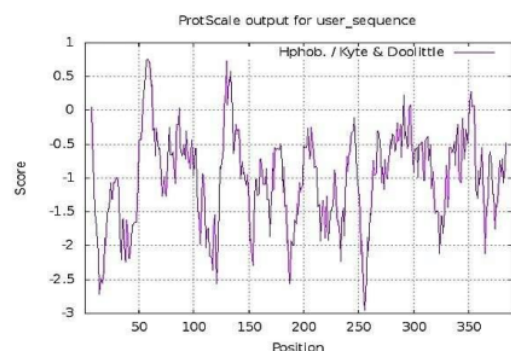
Parameters	Results
Number of amino acids	390
Molecular weight	44738.2
Isoelectric Point Value	9.85
Atomic Composition of C (Carbon)	1961
Atomic Composition of H (Hydrogen)	3181
Atomic Composition of N (Nitrogen)	597
Atomic Composition of O (Oxygen)	577
Atomic Composition of S (Sulfur)	12
Formula	C1961H3181N597O577S12
Number of Atoms	6328
Part Time Estimate	30 hours (mammalian reticulocytes, in vitro)
Instability Index	31.42 (protein stabil)
Aliphatic Index	70.90
Grand Average of Hydrophaticity (GRAVY)	-0.95
Total number of negative residues (Asp + Glu)	48
Total number of positive residues (Arg + Lys)	78

Note: C: Carbon, H: Hydrogen, N: Nitrogen, O: Oxygen, S: Sulfur

The aliphatic index of the 42 kDa subunit pili protein is 70.90 (Table 2), indicating that this protein is predicted to be stable over a wide temperature range (thermostable). This result is consistent with previous research (Gangadhar et al. 2016), which mentions a high aliphatic index. The high range of 74.14 to 80.45 may be stable over a wide temperature range, and (Sivakumar et al. 2007) mention the aliphatic index of antifreeze protein, which ranges from 57.89 to 125.23 based on sequence comparisons of various varieties.

This result shows that proteins with a high number of aliphatic side chains have Ala 4.4%, Val 8.5%, Ile 4.4%, and Leu 6.4% of the AA composition (Table 1). It is expected that Ala, Val, Ile, and Leu will be thermostable (Gasteiger et al. 2005; Gangadhar et al. 2016). The aliphatic index (AI), defined as the relative volume of protein occupied by aliphatic side chains, was thought to be a positive factor in globular protein thermal stability. The GRAVY index of the 42 kDa subunit pili protein is -0.950 (Table 2), where the lower the GRAVY value, the better the protein's interaction with water (Sivakumar et al. 2007). This result was also confirmed using the SOSUI program (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html), which revealed that this protein was water soluble.

Based on the ProtScale program results (Figure 4), the number of graphs that are less than 0 strengthens the results of ProtParam and SOSUI. According to Kyte and Doolittle (1982), the hydrophatic value ranges from -2 to +2, with the higher (positive) hydrophatic value indicating that the protein is more hydrophobic. Hydrophobicity is an important physicochemical property to understand because it has the potential to be exposed to and interact with immunoglobulins during the process of foreign protein expression in the host.

**Figure 4.** Plot graph of the HpHob/Kyte & Doolittle hydrophathy protein of 42 kDa subunit protein *Salmonella typhi*

Secondary structure of the 42 kDa subunit protein analysis

The secondary structure is used to determine the structure and function of the protein, which is dependent on the secondary structure's prediction accuracy. The PSIPRED program on the www.expasy.org website was used to analyze the secondary structure of the 42 kDa subunit pili protein. The protein structure is made up of folded polypeptide chains in the shape of an alpha helix (H), which is a spiral shaped chain of amino acids, a strand/beta sheet (E), which is a chain in the form of sheets composed of amino acids that are bonded to each other through hydrogen bonds, and other structures in the shape of coils or coils (C), which resemble ropes (Figure 5).



Figure 5. Secondary structure of the 42 kDa subunit protein of *Salmonella typhi*

DMPFold Structure

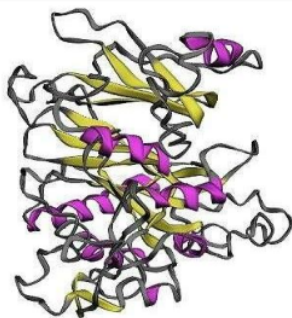


Figure 6. Secondary structure prediction of the 42 kDa subunit protein of *Salmonella typhi*

The results of this study show that the coil strand (elongated strand) dominates the secondary structural elements with a percentage of 38.21% (Figure 5). The coil strand functions in flexibility and change, where the peptide bond is not involved in intra-protein hydrogen bonding, and this structure can interact with water molecules, small ligands, or other proteins (Filiz and Koc 2014). Then comes the alpha helix strand (-helix), which has a percentage of 33.08%, followed by the strand, which has a percentage of 20.77%, and the beta strand (-strand), which has a percentage of 7.95%. With t, the protein structure becomes more stable. The results of this study show an example of protein structure prediction obtained by using DMPFold in the PSIPRED program (Figure 6).

In conclusion, 42 kDa subunit pili protein of *Salmonella typhi* is immunogenic and it can be used to develop a more specific and sensitive diagnostic reagent for typhoid fever.

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