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

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Lampiran 1
Submission

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[biodiv] Submission Acknowledgement

1 message

Ahmad Dwi Setyawan <smujo.id@gmail.com> Thu, Oct 28, 2021 at 3:10 PM
To: Sri Darmawati <ciciekdarma@unimus.ac.id>

Sri Darmawati:

Thank you for submitting the manuscript, "Molecular Characterization of a 42 kDa Subunit Pili Protein of Salmonella typhi Causes Typhoid Fever" to Biodiversitas Journal of Biological Diversity. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

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If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Ahmad Dwi Setyawan

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Note	From
Dr. Sri Darmawati, M.Si Postgraduate of Laboratory for Clinical Science, Universitas Muhammadiyah Semarang, Jl. Kedungmundu Raya No. 18 Semarang 50273, Central Java, Indonesia and Muhammadiyah Research Network for Plasma Medicine (M-Plasmed) Magelang, Indonesia Tel/Fax. +62-812-2503552, ©email: ciciekdarma@unimus.ac.id Editor-in-Chief Biodiversitas Dear Editor-in-Chief: I am pleased to submit an original research article titled "Molecular Characterization of a 42 kDa Subunit Pili Protein of Salmonella typhi Causes Typhoid Fever". by Sri Darmawati, Stalis Norma Ethica, Muhammad Evy Prasetyo, Sulaiman Ngongu Depamede, Eleventi, Mudyawati Kamaruddin for consideration for publication in Biodiversitas. In this manuscript, we 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. We believe that this manuscript is appropriate for publication by Biodiversitas. This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose. Thank you Sincerely, **Dr. Sri Darmawati, M.Si** .	ciciek 2021-10-28 08:06 AM

COVERING LETTER

Dear **Editor-in-Chief,**

I herewith enclosed a research article,

Title:

Molecular Characterization of a 42 kDa Subunit Pili Protein of *Salmonella typhi* Causes Typhoid Fever

Author(s) name:

SRI DARMAWATI[1,2,♥], STALIS NORMA ETHICA[1] , MUHAMMAD EVY PRASETYO[3], SULAIMAN NGONGU DEPAMEDE[4], ELEVENTI[5], MUDYAWATI KAMARUDDIN[1]

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In Indonesia, there are an estimated 800-100,000 cases of typhoid fever, with 91 percent of cases being children aged 3 to 19 year old and 20,000 deaths occurring each year. 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 pilli of *S. typhi* that the findings can be used to develop a typhoid fever diagnostic reagent

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Place and date:

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Sincerely yours,

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Sri Darmawati

Molecular Characterization of a 42 kDa Subunit Pili Protein of *Salmonella typhi* Causes Typhoid Fever

SRI DARMAWATI[1,2,♥], STALIS NORMA ETHICA[1], MUHAMMAD EVY PRASTIYANTO[3], SULAIMAN NGONGU DEPAMEDE[4], ELEVENTI OKTARINA PUTRI[1], MUDYAWATI KAMARUDDIN[1]

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Abstract. In Indonesia, there are an estimated 800-100,000 cases of typhoid fever, with 91 percent of cases being children aged 3 to 19 year old and 20,000 deaths occurring each year. 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 pilli of *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 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.

Key words: Pili protein; *Salmonella typhi*; Major Protein; Typhoid fever

INTRODUCTION

Typhoid fever is an infectious condition that spreads throughout the body and is caused by the bacteria *Salmonella typhi* (*S. typhi*) (Tran et al., 2017; Ajibola et al., 2018). This is one of the leading causes of mortality in many underdeveloped countries, including Indonesia. 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 percent, 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).

As a result, other ways for acquiring specific antigens, such as the 42kDa protein component pili from *Salmonella 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 *Salmonella typhi* by examining physicochemical parameters such as amino acid 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 *Salmonella typhi* strain used in this study was taken from a blood culture of a positive Widal patient. For bacterial identification, API 20E and API 50CHB /E medium (Bio Merieux Inc.) were utilized (Darmawati, et al., 2012). Bacterial cultures were cultured in biphasic medium (BHI Agar slant and BHI broth media) for 48 hours at 37ºC without agitation (Darmawati et al., 2019).

Isolation and Separation of Pili Protein

Pili protein was isolated using the Ehara approach. After 48 hours, bacterial cultures on biphasic media were collected, and a 3% concentration of Tricloro Acetid Acid (TCA) was added (6 mL TCA into 200 mL bacteria), held at room temperature, and centrifuged at 3000 rpm for 20 minutes at 4ºC. 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 4ºC. The resulting suspension was centrifuged for 20 minutes at 4ºC at 3000 rpm, and the supernatant was protein pili.

The pili protein profile was analyzed using the SDS-PAGE 12 percent procedure (Laemli, 1970) stained with 0.25 percent Coomassie Brilliant Blue R250 after purification with 40 percent 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. The sequenced peptide segments were aligned using the Basic Local Alignment Search Tool Protein (BLASTP).

Physicochemical Analysis of 42 kDa Subunit Protein

Expasy ProtParam was used to examine physicochemical data such as amino acid 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 & Doolitle 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 amino acid sequence. PSIPRED features the DMPFold tool, which may be used to see the three-dimensional structure of amino acid 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.

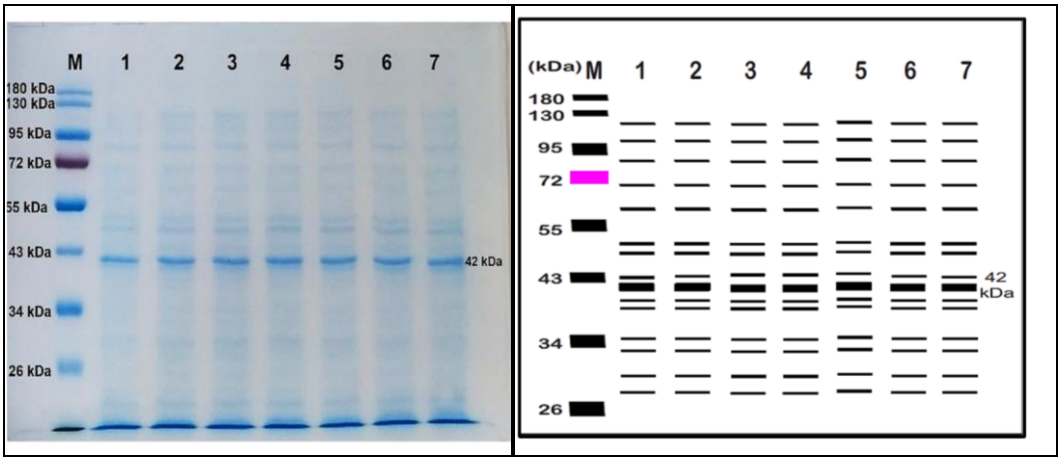

Figure 1. SDS-PAGE analysis of *S.typhi* pili protein (M: Protein markers, 1-7 Pili proteins)

Figure 1 shows the SDS-PAGE results, which show 15 protein bands of varying thicknesses. The protein concentration is indicated by the thickness of the protein band. The thicker the protein band, the higher the concentration (Albert et al, 2002). 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. According to Darmawati (2019), there are two major protein subunits in the pili protein of *S. typhi* isolate BA07.4, namely 42 kDa and 87 kDa, and 14 minor bands using serum extract BHI media, which serves to add nutrients for growth. bacteria and increase the growth rate (Darmawati, S., 2019).

Pili protein 42 kDa is an immunogenic protein based on its protein weight. However, additional research, such as immunoblotting and vaccination tests, are required to prove the imogenicity of this protein. 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 (Tizard, 1987).

Sequencing of *S. typhi* Pili Protein

Sequencing analysis was performed to determine the amino acid 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).

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MIMGVYVDKEHRVKRKSSENGRKSAFAHKVKNGGKNYSRNVQERINRKGASKEVV
VKISGGAVTRQGIRNSIDYMSRESELPVMSESGRVWTGDEILEAKEHMIDRANDP
QHVMNDKGKENKKITQNIVFSPPVSAKVKPEDLLESVRKTMQKKYPNHRFVLGYH
CDKKEHPHVHVVFRIRDNDGKRADIRKKDLREIRTGFCEELKLKGYDVKATHKQQ
HGLNQSVKDAHNTAPKRQKGVYEVVDIGYDHYQNDKTKSKQHFIKLKTLNKGVEK
TYWGADFGDLCSRESVKAGDLVRLKKLGQKEVKIPALDKNGVQHGWKTVHRNEWQ
LENLGVKGVDRTPSASKELVLNSPDMLLKQQQRMAQFTQQKASTLQSEQKLKTGI
KFWGL
```

Figure 2. The 42 kDa subunit pili protein sequence of *S. typhi*

Salmonella enterica bacteria (Accession: EAA0576724.1) had the highest protein homolog value from the alignment results, with a percentage similarity value of 100 percent identity and an E-Value of 0.0, and nine other sequences were *S. enterica* bacteria with an identity similarity percentage value >99 percent 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. (Claverie and Notredame, 2007). The difference in nucleotide and amino acid homology is due to the fact that not all nucleotide changes result in a change in amino acid sequence (Jose et al., 2009).

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 amino acid sequence 12,775 – 13,947 is a protein found in the spore coat (Figure 3).

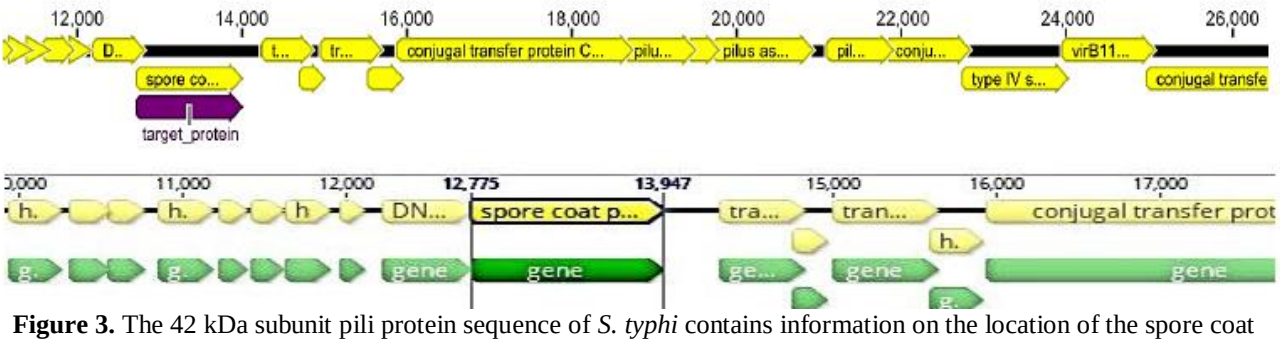

Figure 3. The 42 kDa subunit pili protein sequence of *S. typhi* contains information on the location of the spore coat protein gene (CotH)

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. Alvarez-Fraga et al. (2016) discovered that spore coat proteins are involved in biofilm formation and bacterial attachment to the host in Acinetobacter baumannii strains (Alvarez-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 (Table 1 and Table 2).

Table 1. Amino acid composition of 42 kDa subunit protein

No.	Amino Acid Composition	Percentage (%)	Content
1	Ala (A)	4.4	Netral
2	Arg (R)	6.4	Positif
3	Asn (N)	5.1	Netral
4	Asp (D)	5.9	Negatif
5	Cys (C)	0.8	Netral
6	Gln (Q)	5.6	Netral
7	Glu (E)	6.4	Negatif
8	Gly (G)	7.4	Netral
9	His (H)	4.1	Positif
10	Ile (I)	4.4	Netral
11	Leu (L)	6.4	Netral
12	Lys (K)	13.6	Positif
13	Met (M)	2.3	Netral
14	Phe (F)	2.3	Netral
15	Pro (P)	2.8	Netral
16	Ser (S)	5.9	Netral
17	Thr (T)	3.8	Netral
18	Trp (W)	1.3	Netral
19	Tyr (Y)	2.6	Netral
20	Val (V)	8.5	Netral

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).

Table 2. Physicochemical profile of 42 kDa subunit protein

Parameters	Results
Jumlah asam amino	390
Berat molekul	44738.2
Nilai Titik Iso Elektrik	9.85
Komposisi Atom C (Carbon)	1961
Komposisi Atom H (Hidrogen)	3181
Komposisi Atom N (Nitrogen)	597
Komposisi Atom O (Oksigen)	577
Komposisi Atom S (Sulfur)	12
Formula	C1961H3181N597O577S12
Jumlah Atom	6328
Estimasi Paruh Waktu	30 hours (mammalian reticulocytes, in vitro)
Indeks Instabilitas	31.42 (Protein stabil)

Indeks Aliphatic	70.90
Grand Average of Hydrophaticity (GRAVY)	-0.95
Jumlah total residu negatif (Asp + Glu)	48
Jumlah total residu positif (Arg + Lys)	78

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

According to Tables 1 and 2, the 42 kDa subunit pili protein of S. typhi has 390 aa amino acids with a molecular weight of 44738.20 Daltons and 20 types of amino acids, with lysine (L) being the dominant amino acid and low amino acids 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 (Wilkins et al., 2008).

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, Sivakumar, 2010).

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) mentions the aliphatic index of antifreeze protein, which ranges from 57.89 to 125.23 based on sequence comparisons of various varieties.

Table 1 shows that proteins with a high number of aliphatic side chains have Ala 4.4 percent, Val 8.5 percent, Ilu 4.4 percent, and Leu 6.4 percent of the amino acid composition. It is expected that Ala, Val, Ilu, and Leu will be thermostable (Gangadhar et al, 2016, Gasteiger et al., 2005). 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.

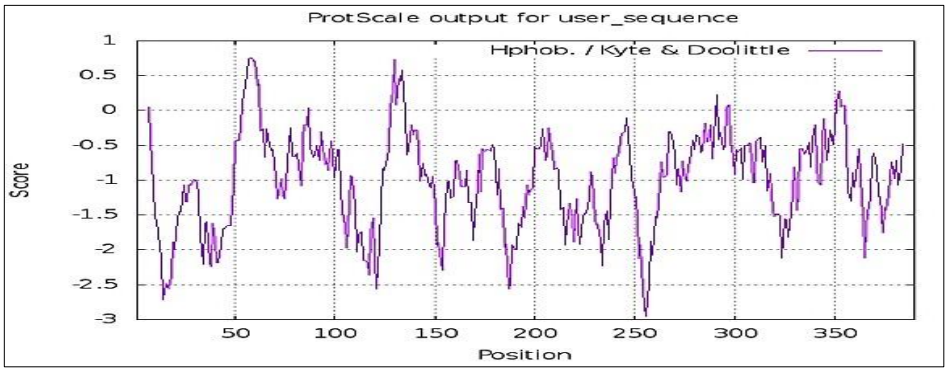

Figure 4. Plot graph of the HpHob./Kyte & Doolitle hydropathy protein of 42 kDa subunit protein *S. typhi*

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 hydropathic value ranges from -2 to +2, with the higher (positive) hydropathic 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.

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).

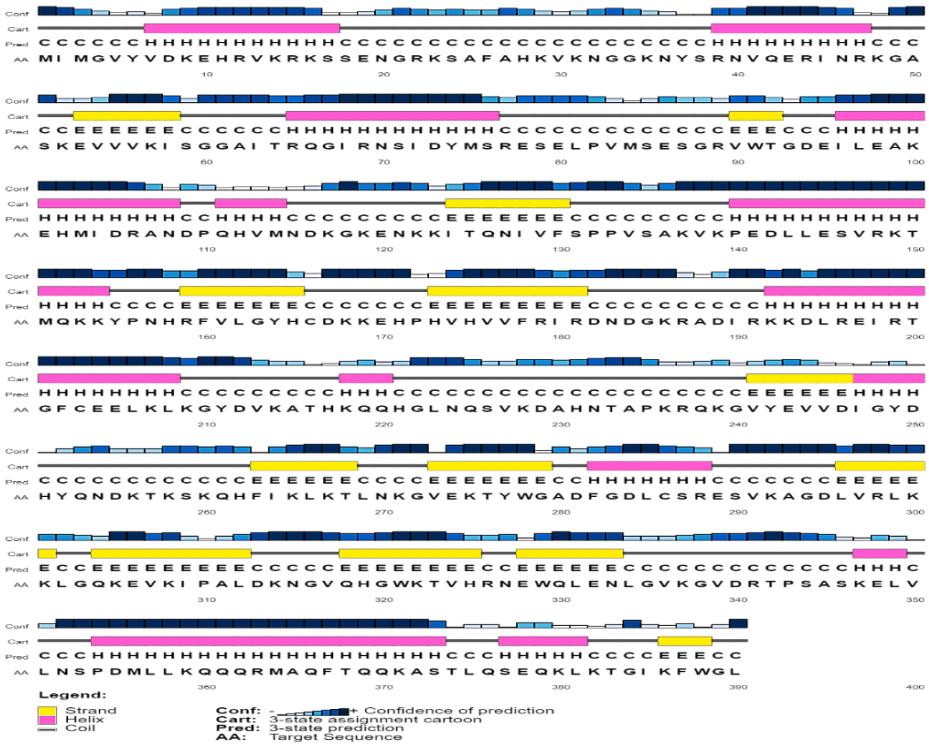

Figure 5. Secondary structure of the 42 kDa subunit protein of *S. typhi*

The results of this study (figure 5) show that the coil strand (elongated strand) dominates the secondary structural elements with a percentage of 38.21 percent. 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; Buxbaum, 2007). Then comes the alpha helix strand (-helix), which has a percentage of 33.08 percent, followed by the strand, which has a percentage of 20.77 percent, and the beta strand (-strand), which has a percentage of 7.95 percent. With t, the protein structure becomes more stable. Figure 6 shows an example of protein structure prediction obtained by using DMPFold in the PSIPRED program.

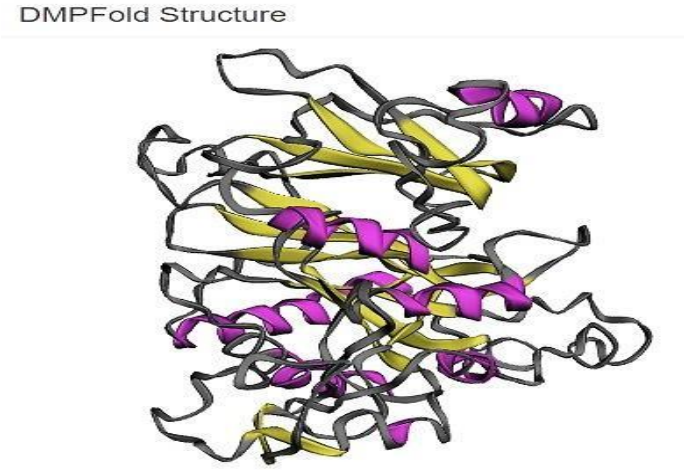

Figure 6. Secondary structure prediction of the 42 kDa subunit protein of *S. typh*

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SUBMISSION CHECKLIST

Ensure that the following items are present:

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Lampiran 2
Pre-Review

Sri Darmawati <ciciekdarma@unimus.ac.id>

[biodiv] New notification from Biodiversitas Journal of Biological Diversity

1 message

Agustina Putri <smujo.id@gmail.com> Thu, Oct 28, 2021 at 4:14 PM
Reply-To: Ahmad Dwi Setyawan <editors@smujo.id>
To: Sri Darmawati <ciciekdarma@unimus.ac.id>

You have a new notification from Biodiversitas Journal of Biological Diversity:

You have been added to a discussion titled "Pre-review" regarding the submission "Molecular Characterization of a 42 kDa Subunit Pili Protein of Salmonella typhi Causes Typhoid Fever".

Link: https://smujo.id/biodiv/authorDashboard/submission/9736

Ahmad Dwi Setyawan

Pre-review

Lampiran 3
Revisi I

Participants

Agustina Putri (aputri1)

Sri Darmawati (ciciek)

Messages

Note	From
Dear Sir/Ma'am, Thank you very much for your manuscript submission. First of all, here we want to inform you that the article which composes 2000-2500 words from introduction to conclusion (table and figure are excluded) will be published as a short-communication. If the authors add other data and compose minimum of 3000 words paper from introduction to conclusion (table and figure are excluded), the article will be published as original full-length paper. Unfortunately, your manuscript does not meet our requirement. This manuscript has outdated references. At least, you need to compose a minimum of 20 references which 80% of scientific journals published in the last 10 years (2011-2021) and maximum 10% of reference in Indonesian. Kindly check, correct accordingly and resubmit your revised paper in this discussion. Thank you, Regards, Agustina Putri	aputri1 2021-10-28 09:13 AM

Revisi I

Participants Edit

Agustina Putri (aputri1)

Sri Darmawati (ciciek)

Lampiran 4
Revised manuscrip

Messages

Note	From
here we attach our research file which has been repaired ciciek, Bu Ciciek_Manuscript_Salmonella typi-UPLOAD.doc	ciciek 2021-11-19 01:16 PM
Dear Sir/Ma'am, Thank you very much for your revised manuscript. Unfortunately, your revised paper still does not meet our requirements. This revised manuscript still has outdated references, you need to compose a minimum of **80% of scientific journals published in the last 10 years (2011-2021).** Please check the attached file below for the detail. Kindly check, correct accordingly and resubmit your revised paper in this discussion. Thank you, Regards, Agustina Putri aputri1, REFERENCES - Darmawati .doc	aputri1 2021-11-22 07:20 AM
Dear editorial team here we attach our revised manuscrip ciciek, revisi II (referensi terbaru).doc	ciciek 2021-11-24 03:42 AM

Sri Darmawati <ciciekdarma@unimus.ac.id>

[biodiv] Editor Decision

2 messages

Agustina Putri <smujo.id@gmail.com> Sun, Jan 9, 2022 at 7:13 PM
To: Sri Darmawati <ciciekdarma@unimus.ac.id>

Sri Darmawati:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Molecular characterization of a 42 kDa subunit pili protein of Salmonella typhi causes typhoid fever".

Our decision is: Revisions Required

Reviewer F:

The manuscript entitled "Molecular characterization of a 42 kDa subunit pili protein of *Salmonella typhi* causes typhoid fever" needs a critical revision for English Language and typo errors.

- Italicize all the scientific names
- with 91 percent of cases being children aged 3 to 19 year old and 20,000 deaths occurring each year…..with 91% of cases being children aged 3 to 19 years…..

- Line 9: Write the full form of *S. typhi* (as occurring first time in abstract)
- Line 11: Write aa as amino acid and follow the same
- Keywords: give other keyword at place of "major protein"
- Line 19: Italicize "enterica"
- Line 22: Write the full form of WHO
- Line 59: Italicize "S.typhi"
- Line 66 characterize the 42kDa subunit pili protein of *Salmonella typhi…….* characterize the 42kDa subunit pili protein of *S. typhi*
- Give the one sentence (last of introduction) what is need of this investigation and what is output that help society
- Line 72: Lowercase bacteria
- The Bacteria *Salmonella typhi* strain….. The bacteria *Salmonella typhi* (Give the strain number here also) as……. The bacteria *Salmonella typhi* strain (…..)….
- Line 73: Don't start the sentence with "For"
- Pili protein was isolated using the Ehara approach…..Give the citation (Ref) here
- Line 83: Correct the spelling of "Tricloro Acetid Acid (TCA)"……trichloroacetic acid
- Line 96-97: The pili protein profile was analyzed using the SDS-PAGE 12 percent procedure…reframe and revise
- 0.25 percent Coomassie Brilliant Blue……0.25 % coomassie brilliant blue
- Follow the same for "percent" as "%" in MS
- Line 106: microwave for 15 seconds. Then incubated for 1 hour…… microwave for 15 seconds and incubated for 1 hour.
- Line 109: Basic Local Alignment Search Tool Protein (BLASTP)…… basic local alignment search tool protein (BLASTp)
- Line 123: Rewrite the sentence
- RESULTS AND DISCUSSIONS…… RESULTS AND DISCUSSION
- The findings of the pili protein isolation were subsequently subjected to SDS-PAGE in order to examine and characterize the pili protein profile (Figure 1)
- Figure 1 shows the SDS-PAGE results, which show 15 protein bands of varying thicknesses…..Reframe and revise and do not write as "figure show" Follow the same in MS
- Line 141-143: According to Darmawati (2019), there are two major protein subunits in the pili protein of *S. typhi* isolate BA07.4, namely 42 kDa and 87 kDa, and 14 minor bands using serum extract BHI media, which serves to add nutrients for growth. bacteria and increase the growth rate (Darmawati, S., 2019). Revise
- Line 152: Reframe the sentence "To obtain….."
- Line 173: Italicize Acinetobacter baumannii

- Line197: Table 1 shows that proteins with a high numbe…..(Revise, please do not start the sentence that Table show or Figure show…….follow the same in whole MS.
- Line 349: Correct the spelling research
- Add the “Conclusion” section- Add one paragraph for the this section.
- Revise the references as per journal instruction… If possible add DOI in each reference

Recommendation: Revisions Required

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

Abstract. ~~In Indonesia, there are an estimated 800-100,000 cases of typhoid fever, with 91 percent of cases being children aged 3 to 19 year old and 20,000 deaths occurring each year.~~ 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 pilli of S*almonella~~.~~ 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 (AA) sequence with a length of 390 AA~~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~~ajor protein~~; 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*) (Tran et al., 2017; Ajibola et al., 2018). Typhoid fever is usually contracted by ingestion of water or food contaminated by faecal 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~~WHO~~ 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 shows 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% ~~percent~~, 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, 2019; Darmawati et al., 2015).

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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 hema-agglutination 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 immonogenic 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 ~~*Salmonella*~~ *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 ~~*Salmonella*~~ *S. typhi* by examining physicochemical parameters such as AA~~amino acid~~ 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 ~~*Salmonella*~~ *S. typhi* strain BA07.4 used in this study was taken from a blood culture of a positive Widal patient. bacterial ~~For bacterial I~~identification use~~,~~ API 20E and API 50CHB /E medium (Bio Merieux Inc.) were utilized (Darmawati, et al., 2012). Bacterial cultures were cultured in biphasic medium (BHI Agar slant and BHI broth media) for 48 hours at 37ºC 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 37ºC with agitation. After that, 50 mL of BHI bacterial culture was added to 500 mL liquid BHI media and incubated for 6 hours at 37ºC 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 37ºC without agitation. The culture is ready to be harvested.

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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~~Tricloro Acetid 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 4ºC. 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 4ºC. The resulting suspension was centrifuged for 20 minutes at 4ºC 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 4ºC. 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 4ºC until completely dissolved. Then the supernatant was centrifuged at 3000 rpm for 20 minutes at 4ºC, 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 ~~12 percent procedure~~ 0.25% coomassie brilliant blue (Banta *et al*, 2021) stained with 0.25~~% percent~~ Coomassie Brilliant Blue R250 after purification with 40~~ percent~~% 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) coomasive brilliant blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid was heated in a microwave for 15 seconds and~~. Then~~ 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 b~~b~~asic local alignment search tool protein (BLASTP).

Physicochemical analysis of 42 kDa subunit protein

Expasy ProtParam was used to examine physicochemical data such as ~~amino acid~~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 & Doolitle 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 ~~amino acid~~AA sequence. PSIPRED features the DMPFold tool, which may be used to see the three-dimensional structure of ~~amino acid~~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.

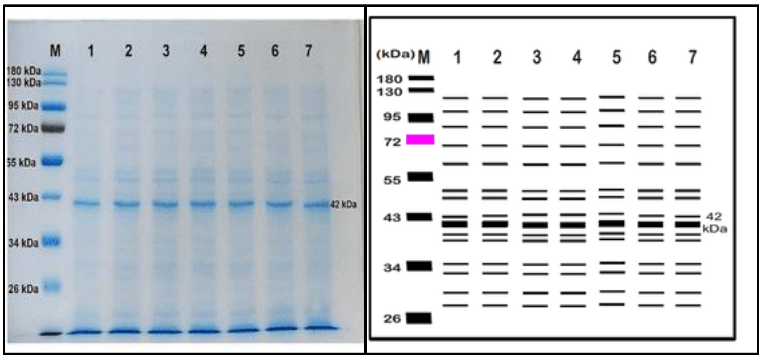

Figure 1. SDS-PAGE analysis of *S.typhi* pili protein (M: Protein markers, 1-7 Pili proteins)

~~Figure 1 shows the~~The SDS-PAGE results showing~~,~~ which show 15 protein bands of varying thicknesses. The protein concentration is indicated by the thickness of the protein band. The thicker the protein band, the higher the concentration (Sulistyarsi 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. According to Darmawati (2019), there are two major protein subunits in the pili protein of *S. typhi* isolate BA07.4, namely 42 kDa and 87 kDa, and 14 minor bands using serum extract BHI media, which serves to add nutrients for growth. bacteria and increase the growth rate (Darmawati, S., 2019).

Pili protein 42 kDa is an immunogenic protein based on its protein weight. However, additional research, such as immunoblotting and vaccination tests, are required to prove the imogenicity of this protein. 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).

Sequencing of *S. typhi* Pili protein

Sequencing analysis was performed to determine the ~~amino acid~~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).

MIMGVYVDKEHRVKRKSSENGRKSAFAHKVKNGGKNYSRNVQERINRKGASKEVV
VKISGGAVTRQGIRNSIDYMSRESELPVMSESGRVWTGDEILEAKEHMIDRANDP
QHVMNDKGKENKKITQNIVFSPPVSAKVKPEDLLESVRKTMQKKYPNHRFVLGYH
CDKKEHPHVHVVFRIRDNDGKRADIRKKDLREIRTGFCEELKLKGYDVKATHKQQ
HGLNQSVKDAHNTAPKRQKGVYEVVDIGYDHYQNDKTKSKQHFIKLKTLNKGVEK
TYWGADFGDLCSRESVKAGDLVRLKKLGQKEVKIPALDKNGVQHGWKTVHRNEWQ
LENLGVKGVDRTPSASKELVLNSPDMLLKQQQRMAQFTQQKASTLQSEQKLKTGI
KFWGL

Figure 2. The 42 kDa subunit pili protein sequence of *S. typhi*

Salmonella enterica bacteria (Accession: EAA0576724.1) had the highest protein homolog value from the alignment results, with a percentage similarity value of 100 ~~percent~~% identity and an E-Value of 0.0, and nine other sequences were *S. enterica* bacteria with an identity similarity percentage value >99 ~~percent~~% 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 ~~amino acid~~AA homology is due to the fact that not all nucleotide changes result in a change in ~~amino acid~~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 ~~amino acid~~AA sequence 12,775 – 13,947 is a protein found in the spore coat (Figure 3).

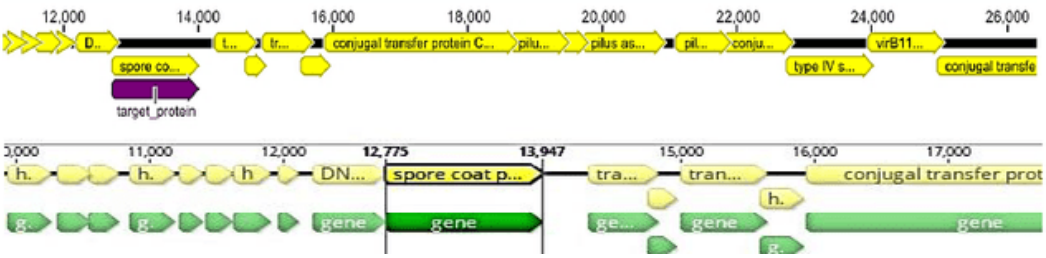

Figure 3. The 42 kDa subunit pili protein sequence of *S. typhi* contains information on the location of the spore coat protein gene (CotH)

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. Alvarez-Fraga et al. (2016) discovered that spore coat proteins are involved in biofilm formation and bacterial attachment to the host in *Acinetobacter baumannii* strains (Alvarez-Fraga et al., 2016).

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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 (Table 1 and Table 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 aa ~~amino acid~~AAs with a molecular weight of 44738.20 Daltons and 20 types of ~~amino acid~~AAs, with lysine (L) being the dominant ~~amino acid~~AA and low

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~~amino acid~~AAs 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).

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) mentions the aliphatic index of antifreeze protein, which ranges from 57.89 to 125.23 based on sequence comparisons of various varieties.

~~Table 1~~This result shows that proteins with a high number of aliphatic side chains have Ala 4.4~~ percent~~%, Val 8.5 ~~percent~~%, Ilu 4.4~~ percent~~%, and Leu 6.4~~ percent~~ % of the ~~amino acid~~AA composition (Table 1). It is expected that Ala, Val, Ilu, and Leu will be thermostable (Gangadhar et al, 2016, Gasteiger et al., 2005). 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.

Table 1. ~~Amino acid~~AA composition of 42 kDa subunit protein

No.	~~Amino Acid~~AA Composition	Percentage (%)	Content
1	Ala (A)	4.4	Netral
2	Arg (R)	6.4	Positif
3	Asn (N)	5.1	Netral
4	Asp (D)	5.9	Negatif
5	Cys (C)	0.8	Netral
6	Gln (Q)	5.6	Netral
7	Glu (E)	6.4	Negatif
8	Gly (G)	7.4	Netral
9	His (H)	4.1	Positif
10	Ile (I)	4.4	Netral
11	Leu (L)	6.4	Netral
12	Lys (K)	13.6	Positif
13	Met (M)	2.3	Netral
14	Phe (F)	2.3	Netral
15	Pro (P)	2.8	Netral
16	Ser (S)	5.9	Netral
17	Thr (T)	3.8	Netral
18	Trp (W)	1.3	Netral
19	Tyr (Y)	2.6	Netral
20	Val (V)	8.5	Netral

Table 2. Physicochemical profile of 42 kDa subunit protein

Parameters	Results
Jumlah asam amino	390
Berat molekul	44738.2
Nilai Titik Iso Elektrik	9.85
Komposisi Atom C (Carbon)	1961
Komposisi Atom H (Hidrogen)	3181
Komposisi Atom N (Nitrogen)	597
Komposisi Atom O (Oksigen)	577
Komposisi Atom S (Sulfur)	12
Formula	C1961H3181N597O577S12
Jumlah Atom	6328
Estimasi Paruh Waktu	30 hours (mammalian reticulocytes, in vitro)

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Indeks Instabilitas	31.42 (Protein stabil)
Indeks Aliphatic	70.90
Grand Average of Hydrophaticity (GRAVY)	-0.95
Jumlah total residu negatif (Asp + Glu)	48
Jumlah total residu positif (Arg + Lys)	78

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

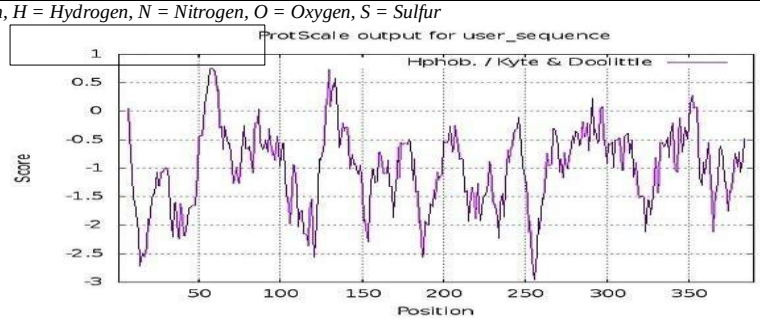

Figure 4. Plot graph of the HpHob./Kyte & Doolitle hydropathy protein of 42 kDa subunit protein *S. typhi*

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 hydropathic value ranges from -2 to +2, with the higher (positive) hydropathic 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.

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 acid~~AAs, a strand/beta sheet (E), which is a chain in the form of sheets composed of ~~amino acid~~AAs 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).

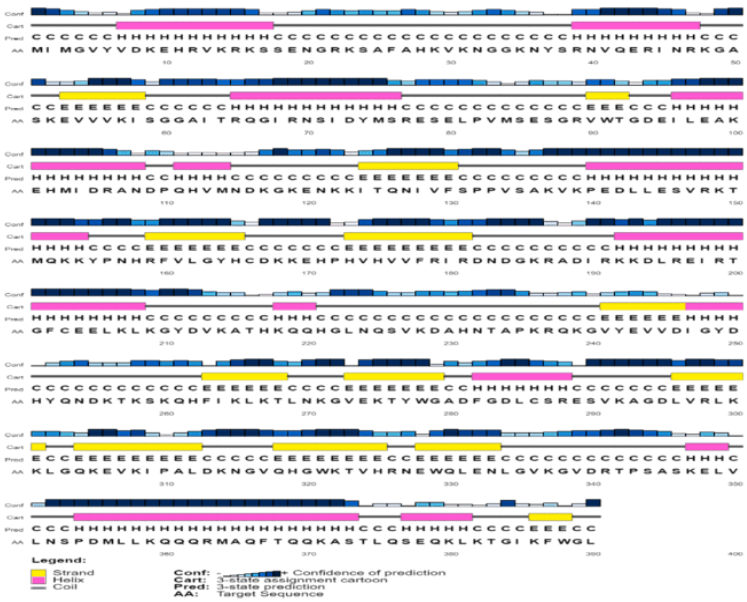

Figure 5. Secondary structure of the 42 kDa subunit protein of *S. typhi*

The results of this study ~~(figure 5)~~ show that the coil strand (elongated strand) dominates the secondary structural elements with a percentage of 38.21~~ percent~~% (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% ~~percent~~, followed by the strand, which has a percentage of 20.77~~ percent~~%, and the beta strand (-strand), which has a percentage of 7.95% ~~percent~~. With t, the protein structure becomes more stable. The results of this study ~~Figure 6~~ shows an example of protein structure prediction obtained by using DMPFold in the PSIPRED program (Figure 6).

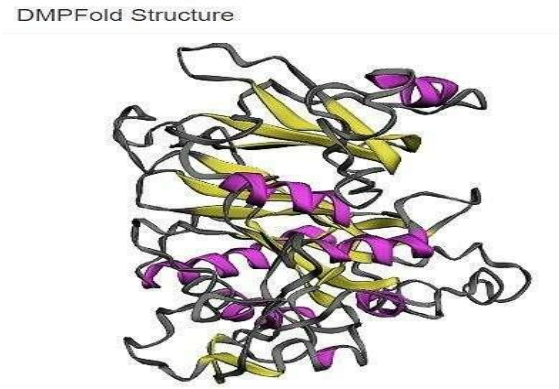

Figure 6. Secondary structure prediction of the 42 kDa subunit protein of *S. typh*

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.

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ACKNOWLEDGEMENTS

We acknowledge the support of the Ministry of Science and Technology / National Agency for Research and Innovation of the Republic of Indonesia and the Ministry of Education ((RISTEK/BRIN) Grant No SP DIPA-023.17.1.690439/2021 We also thanks to Universitas Muhammadiyah Semarang for facilitated this research

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Respon to Reviewer 2

Sri Darmawati <ciciekdarma@unimus.ac.id>

[biodiv] Editor Decision

1 message

Smujo Editors <smujo.id@gmail.com> Tue, Jan 11, 2022 at 3:18 PM
To: Sri Darmawati <ciciekdarma@unimus.ac.id>

Sri Darmawati:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Molecular characterization of a 42 kDa subunit pili protein of Salmonella typhi causes typhoid fever".

Our decision is: Revisions Required

Reviewer A:

Dear Author

Revision of MS is okay
There are some more corrections required, please see the attached

Recommendation: Revisions Required

Biodiversitas Journal of Biological Diversity

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

Abstract. 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 pilli 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*) (Tran et al., 2017; Ajibola et al., 2018). Typhoid fever is usually contracted by ingestion of water or food contaminated by faecal 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 shows 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, 2019; Darmawati et al., 2015).

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 hema-agglutination 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 immonogenic 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~~ 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 37ºC 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 37ºC with agitation. After that, 50 mL of BHI bacterial culture was added to 500 mL liquid BHI media and incubated for 6 hours at 37ºC 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 37ºC 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 4ºC. 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 4ºC. The resulting suspension was centrifuged for 20 minutes at 4ºC 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 4ºC. 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 4ºC until completely dissolved. Then the supernatant was centrifuged at 3000 rpm for 20 minutes at 4ºC, 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) coomasive 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

Expasy 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 & Doolitle 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.

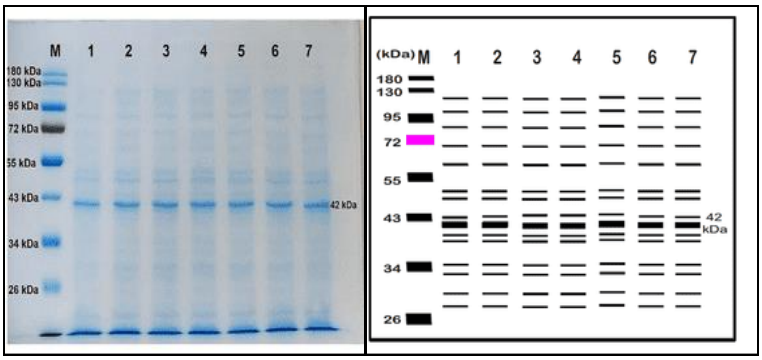

Figure 1. SDS-PAGE analysis of *S.typhi* pili protein (M: Protein markers, 1-7 Pili proteins)

The SDS-PAGE results showing~~, which 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 (Sulistyarsi 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).

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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).

MIMGVYVDKEHRVKRKSSENGRKSAFAHKVKNGGKNYSRNVQERINRKGASKEVVVKISGGAITRQGIRN
SIDYMSRESELPVMSESGRVWTGDEILEAKEHMIDRANDPQHVMNDKGKENKKITQNIVFSPPVSAKVKP
EDLLESVRKTMQKKYPNHRFVLGYHCDKKEHPHVHVVFRIRDNDGKRADIRKKDLREIRTGFCEELKLKG
YDVKATHKQQHGLNQSVKDAHNTAPKRQKGVYEVVDIGYDHYQNDKTKSKQHFIKLKTLNKGVEKTYWGA
DFGDLCSRESVKAGDLVRLKKLGQKEVKIPALDKNGVQHGWKTVHRNEWQLENLGVKGVDRTPSASKELV
LNSPDMLLKQQQRMAQFTQQKASTLQSEQKLKTGIKFWGL

MIMGVYVDKEHRVKRKSSENGRKSAFAHKVKNGGKNYSRNVQERINRKGASKEVV
VKISGGAVTRQGIRNSIDYMSRESELPVMSESGRVWTGDEILEAKEHMIDRANDP
QHVMNDKGKENKKITQNIVFSPPVSAKVKPEDLLESVRKTMQKKYPNHRFVLGYH
CDKKEHPHVHVVFRIRDNDGKRADIRKKDLREIRTGFCEELKLKGYDVKATHKQQ
HGLNQSVKDAHNTAPKRQKGVYEVVDIGYDHYQNDKTKSKQHFIKLKTLNKGVEK
TYWGADFGDLCSRESVKAGDLVRLKKLGQKEVKIPALDKNGVQHGWKTVHRNEWQ
LENLGVKGVDRTPSASKELVLNSPDMLLKQQQRMAQFTQQKASTLQSEQKLKTGI
KFWGL

Figure 2. The 42 kDa subunit pili protein sequence of *S. typhi*

Salmonella enterica ~~bacteria~~ (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).

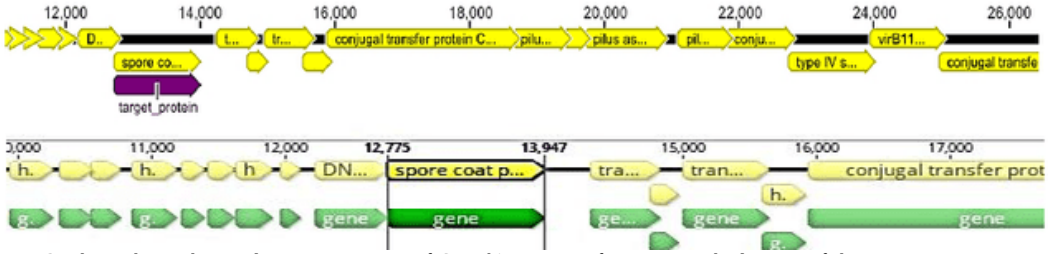

Figure 3. The 42 kDa subunit pili protein sequence of *S. typhi* contains information on the location of the spore coat protein gene (CotH)

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. Alvarez-Fraga et al. (2016) discovered that spore coat proteins are involved in biofilm formation and bacterial attachment to the host in *Acinetobacter baumannii* strains (Alvarez-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 (Table 1 and Table 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,

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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).

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) mentions 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%, Ilu 4.4%, and Leu 6.4% of the AA composition (Table 1). It is expected that Ala, Val, Ilu, and Leu will be thermostable (Gangadhar et al, 2016, Gasteiger et al., 2005). 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.

Table 1. Amino acid composition of 42 kDa subunit protein

No.	Amino Acid Composition	Percentage (%)	Content
1	Ala (A)	4.4	Netral
2	Arg (R)	6.4	Positif
3	Asn (N)	5.1	Netral
4	Asp (D)	5.9	Negatif
5	Cys (C)	0.8	Netral
6	Gln (Q)	5.6	Netral
7	Glu (E)	6.4	Negatif
8	Gly (G)	7.4	Netral
9	His (H)	4.1	Positif
10	Ile (I)	4.4	Netral
11	Leu (L)	6.4	Netral
12	Lys (K)	13.6	Positif
13	Met (M)	2.3	Netral
14	Phe (F)	2.3	Netral
15	Pro (P)	2.8	Netral
16	Ser (S)	5.9	Netral
17	Thr (T)	3.8	Netral
18	Trp (W)	1.3	Netral
19	Tyr (Y)	2.6	Netral
20	Val (V)	8.5	Netral

Table 2. Physicochemical profile of 42 kDa subunit protein

Parameters	Results
Jumlah asam amino	390
Berat molekul	44738.2
Nilai Titik Iso Elektrik	9.85
Komposisi Atom C (Carbon)	1961
Komposisi Atom H (Hidrogen)	3181
Komposisi Atom N (Nitrogen)	597
Komposisi Atom O (Oksigen)	577
Komposisi Atom S (Sulfur)	12
Formula	C1961H3181N597O577S12
Jumlah Atom	6328
Estimasi Paruh Waktu	30 hours (mammalian reticulocytes, in vitro)
Indeks Instabilitas	31.42 (Protein stabil)
Indeks Aliphatic	70.90

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Grand Average of Hydrophaticity (GRAVY)	-0.95
Jumlah total residu negatif (Asp + Glu)	48
Jumlah total residu positif (Arg + Lys)	78

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

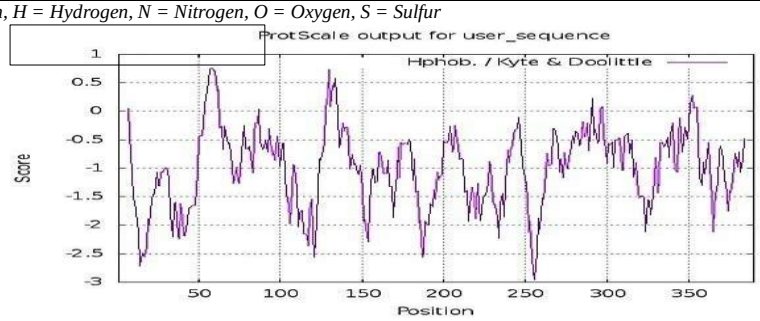

Figure 4. Plot graph of the HpHob./Kyte & Doolitle hydropathy protein of 42 kDa subunit protein *S. typhi*

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 hydropathic value ranges from -2 to +2, with the higher (positive) hydropathic 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.

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).

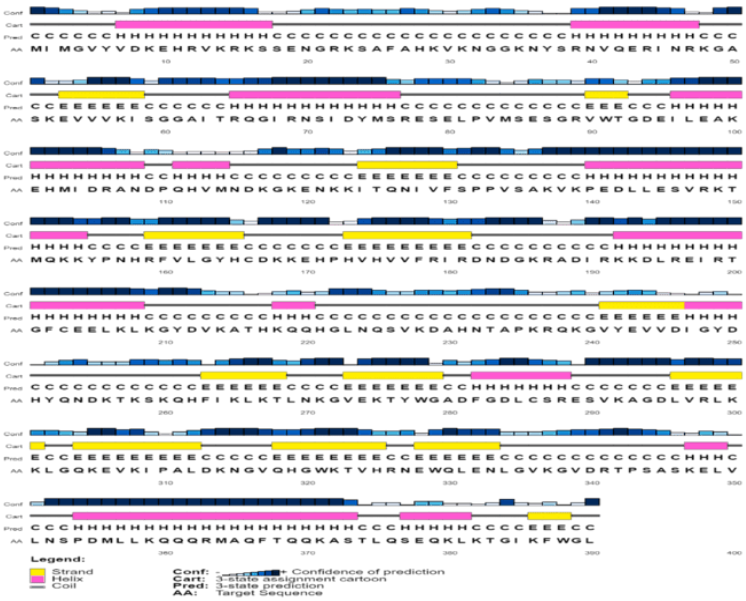

Figure 5. Secondary structure of the 42 kDa subunit protein of *S. typhi*

The results of this study show that the coil strand (elongated strand) dominates the secondary structural elements with a percentage of 38.21% (~~figure~~ 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 shows an example of protein structure prediction obtained by using DMPFold in the PSIPRED program (Figure 6).

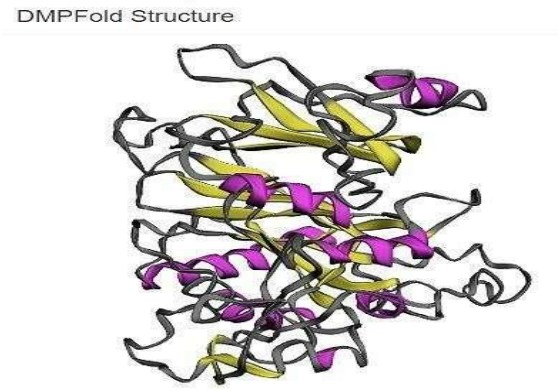

Figure 6. Secondary structure prediction of the 42 kDa subunit protein of *S. typh*

concluded that this 42 kDa subunit pili protein of Salmonella typhi is immunogenic and that it can be used to develop a more specific and sensitive diagnostic reagent for typhoid fever

~~**In conclusion, …….**~~

~~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~~

ACKNOWLEDGEMENTS

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

Abstract. 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 pilli 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 AA (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*) (Tran et al., 2017; Ajibola et al., 2018). Typhoid fever is usually contracted by ingestion of water or food contaminated by faecal 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 shows 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, 2019; Darmawati et al., 2015).

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 hema-agglutination 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 immonogenic 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. bacterial Identification use API 20E and API 50CHB /E medium (Bio Merieux Inc.) were utilized (Darmawati, et al., 2012). Bacterial cultures were cultured in biphasic medium (BHI Agar slant and BHI broth media) for 48 hours at 37ºC 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 37ºC with agitation. After that, 50 mL of BHI bacterial culture was added to 500 mL liquid BHI media and incubated for 6 hours at 37ºC 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 37ºC 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 4ºC. 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 4ºC. The resulting suspension was centrifuged for 20 minutes at 4ºC 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 4ºC. 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 4ºC until completely dissolved. Then the supernatant was centrifuged at 3000 rpm for 20 minutes at 4ºC, 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) coomasive 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

Expasy 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 & Doolitle 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.

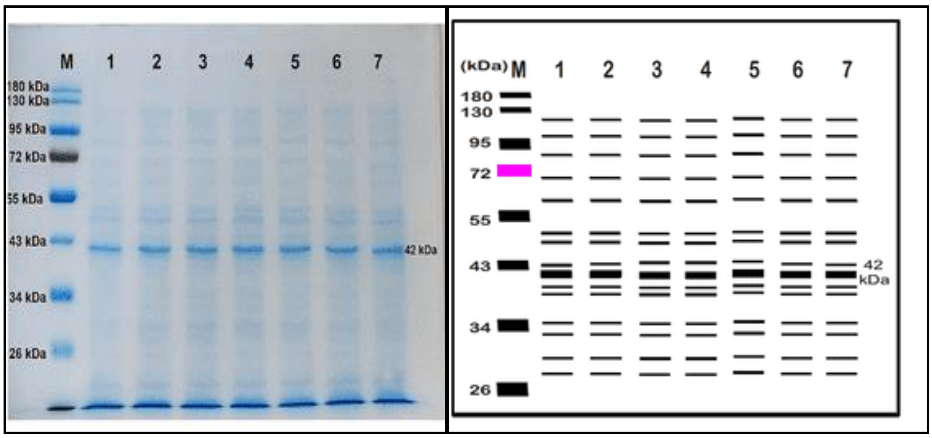

Figure 1. SDS-PAGE analysis of *S.typhi* pili protein (M: Protein markers, 1-7 Pili proteins)

The SDS-PAGE results showing which show 15 protein bands of varying thicknesses. The protein concentration is indicated by the thickness of the protein band. The thicker the protein band, the higher the concentration (Sulistyarsi 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. According to Darmawati (2019), there are two major protein subunits in the pili protein of *S. typhi* isolate BA07.4, namely 42 kDa and 87 kDa, and 14 minor bands using serum extract BHI media, which serves to add nutrients for growth. bacteria and increase the growth rate (Darmawati, S., 2019).

Pili protein 42 kDa is an immunogenic protein based on its protein weight. However, additional research, such as immunoblotting and vaccination tests, are required to prove the imogenicity of this protein. 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).

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).

MIMGVYVDKEHRVKRKSSENGRKSAFAHKVKNGGKNYSRNVQERINRKGASKEVV
VKISGGAVTRQGIRNSIDYMSRESELPVMSESGRVWTGDEILEAKEHMIDRANDP
QHVMNDKGKENKKITQNIVFSPPVSAKVKPEDLLESVRKTMQKKYPNHRFVLGYH
CDKKEHPHVHVVFRIRDNDGKRADIRKKDLREIRTGFCEELKLKGYDVKATHKQQ
HGLNQSVKDAHNTAPKRQKGVYEVVDIGYDHYQNDKTKSKQHFIKLKTLNKGVEK
TYWGADFGDLCSRESVKAGDLVRLKKLGQKEVKIPALDKNGVQHGWKTVHRNEWQ
LENLGVKGVDRTPSASKELVLNSPDMLLKQQQRMAQFTQQKASTLQSEQKLKTGI
KFWGL

Figure 2. The 42 kDa subunit pili protein sequence of *S. typhi*

Salmonella enterica bacteria (Accession: 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).

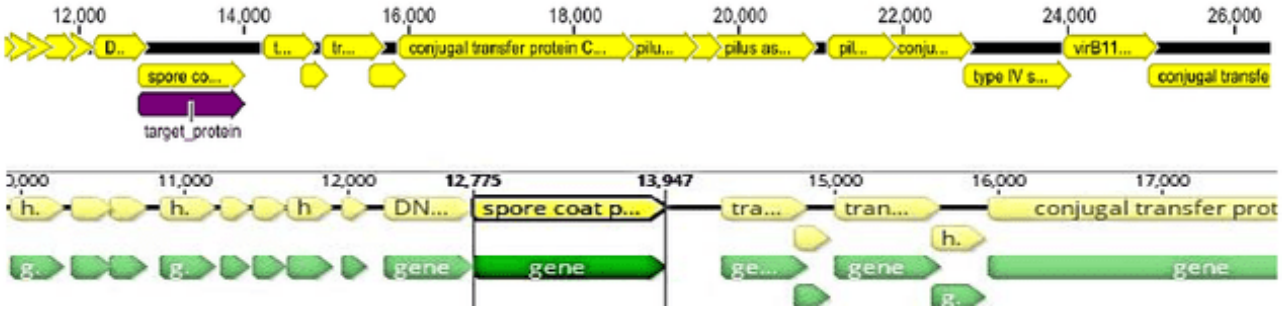

Figure 3. The 42 kDa subunit pili protein sequence of *S. typhi* contains information on the location of the spore coat protein gene (CotH)

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. Alvarez-Fraga et al. (2016) discovered that spore coat proteins are involved in biofilm formation and bacterial attachment to the host in *Acinetobacter baumannii* strains (Alvarez-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 (Table 1 and Table 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 aa AAs with a molecular weight of 44738.20 Daltons and 20 types of AAs, with lysine (L) being the dominant AA and low AAs 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).

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) mentions 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%, Ilu 4.4%, and Leu 6.4% of the AA composition (Table 1). It is expected that Ala, Val, Ilu, and Leu will be thermostable (Gangadhar et al, 2016, Gasteiger et al., 2005). 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.

Table 1. AA composition of 42 kDa subunit protein

No.	AA Composition	Percentage (%)	Content
1	Ala (A)	4.4	Netral
2	Arg (R)	6.4	Positif
3	Asn (N)	5.1	Netral
4	Asp (D)	5.9	Negatif
5	Cys (C)	0.8	Netral
6	Gln (Q)	5.6	Netral
7	Glu (E)	6.4	Negatif
8	Gly (G)	7.4	Netral
9	His (H)	4.1	Positif
10	Ile (I)	4.4	Netral
11	Leu (L)	6.4	Netral
12	Lys (K)	13.6	Positif
13	Met (M)	2.3	Netral
14	Phe (F)	2.3	Netral
15	Pro (P)	2.8	Netral
16	Ser (S)	5.9	Netral
17	Thr (T)	3.8	Netral
18	Trp (W)	1.3	Netral
19	Tyr (Y)	2.6	Netral
20	Val (V)	8.5	Netral

Table 2. Physicochemical profile of 42 kDa subunit protein

Parameters	Results
Jumlah asam amino	390
Berat molekul	44738.2
Nilai Titik Iso Elektrik	9.85
Komposisi Atom C (Carbon)	1961
Komposisi Atom H (Hidrogen)	3181
Komposisi Atom N (Nitrogen)	597
Komposisi Atom O (Oksigen)	577
Komposisi Atom S (Sulfur)	12
Formula	C1961H3181N597O577S12
Jumlah Atom	6328
Estimasi Paruh Waktu	30 hours (mammalian reticulocytes, in vitro)
Indeks Instabilitas	31.42 (Protein stabil)
Indeks Aliphatic	70.90
Grand Average of Hydrophaticity (GRAVY)	-0.95
Jumlah total residu negatif (Asp + Glu)	48
Jumlah total residu positif (Arg + Lys)	78

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

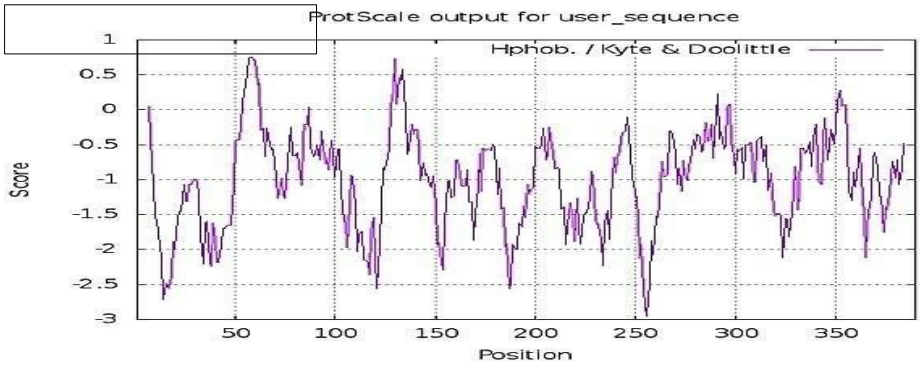

Figure 4. Plot graph of the HpHob./Kyte & Doolitle hydropathy protein of 42 kDa subunit protein *S. typhi*

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 hydropathic value ranges from -2 to +2, with the higher (positive) hydropathic 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.

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 AAs, a strand/beta sheet (E), which is a chain in the form of sheets composed of AAs 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 *S. 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 shows an example of protein structure prediction obtained by using DMPFold in the PSIPRED program (Figure 6).

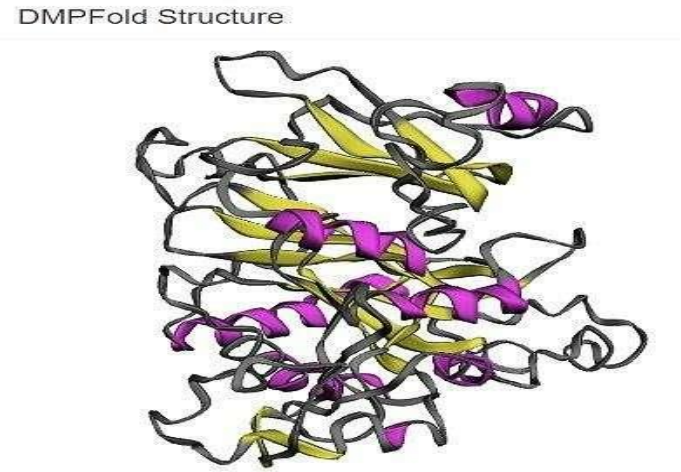

Figure 6. Secondary structure prediction of the 42 kDa subunit protein of *S. typh*

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

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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: xxxx.* 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 pilli 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 faecal 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 shows 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. 2019; Darmawati et al. 2015).

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 hema-agglutination 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 immonogenic 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 37ºC 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 37ºC with agitation. After that, 50 mL of BHI bacterial culture was added to 500 mL liquid BHI media and incubated for 6 hours at 37ºC 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 37ºC 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 4ºC. 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 4ºC. The resulting suspension was centrifuged for 20 minutes at 4ºC 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 4ºC. 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 4ºC until completely dissolved. Then the supernatant was centrifuged at 3000 rpm for 20 minutes at 4ºC, 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) coomasive 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

Expasy 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 & Doolitle 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 showing 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 (Sulistyarsi 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).

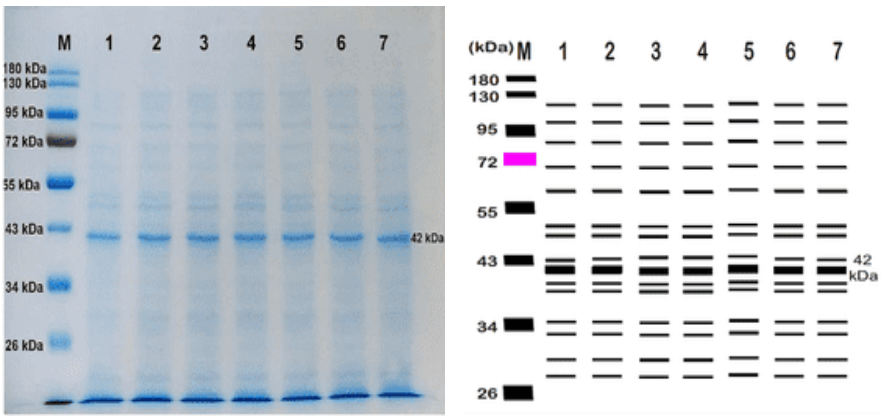

Figure 1. SDS-PAGE analysis of *S. typhi* pili protein. M: Protein markers. 1-7: Pili proteins

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 (Table 1 and Table 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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MIMGVYVDKEHRVKRKSSENGRKSAFAHKVKNGGKNYSRNVQERINRKGASKEVVVKISGGAITRQGIRN
SIDYMSRESELPVMSESGRVWTGDEILEAKEHMIDRANDPQHVMNDKGKENKKITQNIVFSPPVSAKVKP
EDLLESVRKTMQKKYPNHRFVLGYHCDKKEHPHVHVVFRIRDNDGKRADIRKKDLREIRTGFCEELKLKG
YDVKATHKQQHGLNQSVKDAHNTAPKRQKGVYEVVDIGYDHYQNDKTKSKQHFIKLKTLNKGVEKTYWGA
DFGDLCSRESVKAGDLVRLKKLGQKEVKIPALDKNGVQHGWKTVHRNEWQLENLGVKGVDRTPSASKELV
LNSPDMLLKQQQRMAQFTQQKASTLQSEQKLKTGIKFWGL
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Figure 2. The 42 kDa subunit pili protein sequence of *S. typhi*

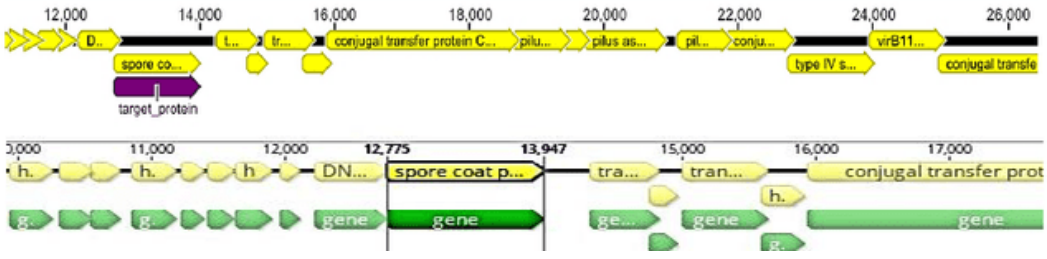

Figure 3. The 42 kDa subunit pili protein sequence of *S. typhi* contains information on the location of the spore coat protein gene (CotH)

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
Jumlah asam amino	390
Berat molekul	44738.2
Nilai Titik Iso Elektrik	9.85
Komposisi Atom C (Carbon)	1961
Komposisi Atom H (Hidrogen)	3181
Komposisi Atom N (Nitrogen)	597
Komposisi Atom O (Oksigen)	577
Komposisi Atom S (Sulfur)	12
Formula	C1961H3181N597O577S12
Jumlah Atom	6328
Estimasi Paruh Waktu	30 hours (mammalian reticulocytes, in vitro)
Indeks Instabilitas	31.42 (Protein stabil)
Indeks Aliphatic	70.90
Grand Average of Hydrophaticity (GRAVY)	-0.95
Jumlah total residu negatif (Asp + Glu)	48
Jumlah total residu positif (Arg + Lys)	78

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

Comment [MOU1]: still in Indonesian

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) mentions 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%, Ilu 4.4%, and Leu 6.4% of the AA composition (Table 1). It is expected that Ala, Val, Ilu, and Leu will be thermostable (Gangadhar et al. 2016, Gasteiger et al. 2005). 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 hydropathic value ranges from -2 to +2, with the higher (positive) hydropathic 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.

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).

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 that it can be used to develop a more specific and sensitive diagnostic reagent for typhoid fever.

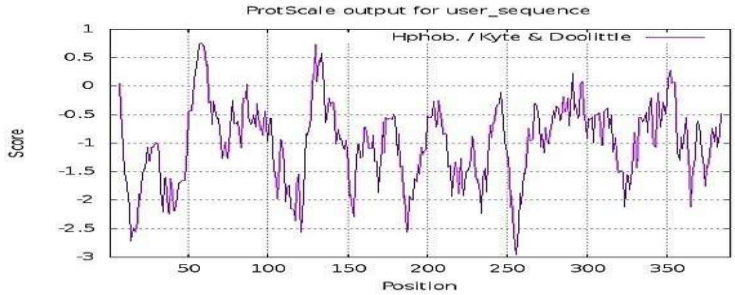

Figure 4. Plot graph of the HpHob./Kyte & Doolitle hydropathy protein of 42 kDa subunit protein *S. typhi*

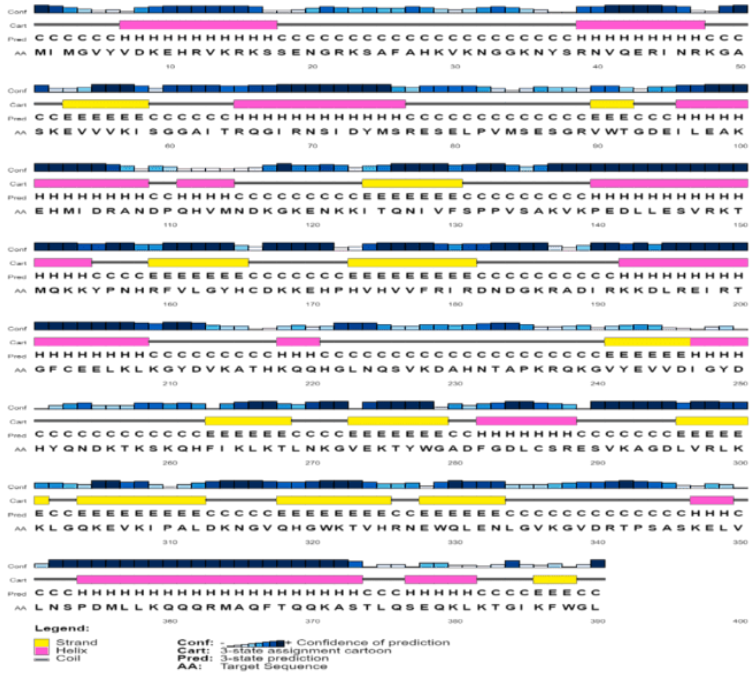

Figure 5. Secondary structure of the 42 kDa subunit protein of *S. typhi*

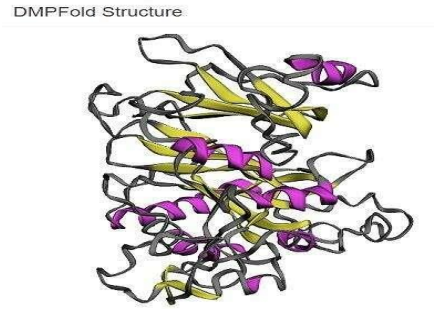

Figure 6. Secondary structure prediction of the 42 kDa subunit protein of *S. typi*

ACKNOWLEDGEMENTS

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[biodiv] Editor Decision

1 message

Agustina Putri <smujo.id@gmail.com> Fri, Feb 4, 2022 at 9:24 PM
To: SRI DARMAWATI <ciciekdarma@unimus.ac.id>, STALIS NORMA ETHICA <authors@smujo.id>

SRI DARMAWATI, STALIS NORMA ETHICA, MUHAMMAD EVY PRASTIYANTO, SULAIMAN NGONGU DEPAMEDE, ELEVENTI OKTARINA PUTRI, MUDYAWATI KAMARUDDIN:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Molecular characterization of a 42 kDa subunit pili protein of Salmonella typhi causes typhoid fever".

Our decision is to: Accept Submission

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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 pilli 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 hema-agglutination 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 37ºC 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 37ºC with agitation. After that, 50 mL of BHI bacterial culture was added to 500 mL liquid BHI media and incubated for 6 hours at 37ºC 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 37ºC 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 4ºC. 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 4ºC. The resulting suspension was centrifuged for 20 minutes at 4ºC 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 4ºC. 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 4ºC until completely dissolved. Then the supernatant was centrifuged at 3000 rpm for 20 minutes at 4ºC, 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) coomasive 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

Expasy 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 & Doolitle 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 (Sulistyarsi 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).

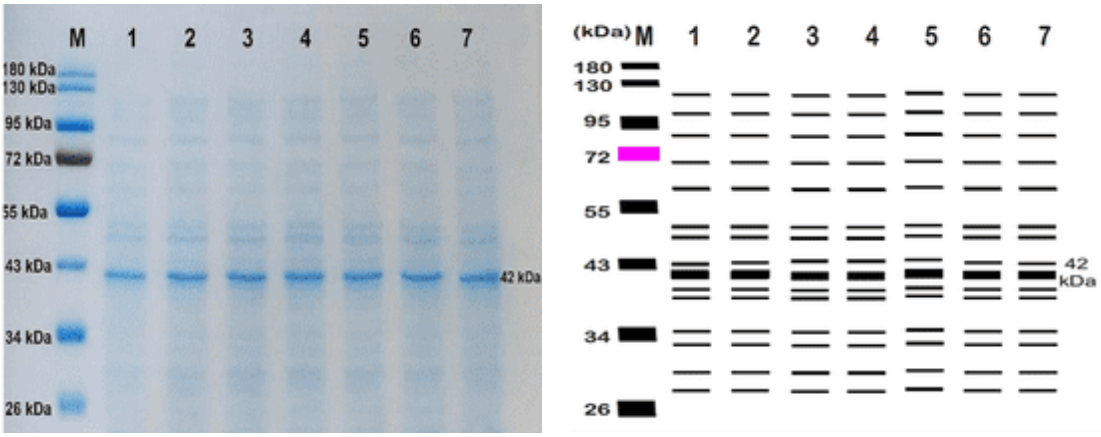

Figure 1. SDS-PAGE analysis of *Salmonella typhi* pili protein. M: Protein markers. 1-7: Pili proteins

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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MIMGVYVDKEHRVKRKSSENGRKSAFAHKVKNGGKNYSRNVQERINRKGASKEVVVKISGGAITRQGIRN
SIDYMSRESELPVMSESGRVWTGDEILEAKEHMIDRANDPQHVMNDKGKENKKITQNIVFSPPVSAKVKP
EDLLESVRKTMQKKYPNHRFVLGYHCDKKEHPHVHVVFRIRDNDGKRADIRKKDLREIRTGFCEELKLKG
YDVKATHKQQHGLNQSVKDAHNTAPKRQKGVYEVVDIGYDHYQNDKTKSKQHFIKLKTLNKGVEKTYWGA
DFGDLCSRESVKAGDLVRLKKLGQKEVKIPALDKNGVQHGWKTVHRNEWQLENLGVKGVDRTPSASKELV
LNSPDMLLKQQQRMAQFTQQKASTLQSEQKLKTGIKFWGL
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Figure 2. The 42 kDa subunit pili protein sequence of *Salmonella typhi*

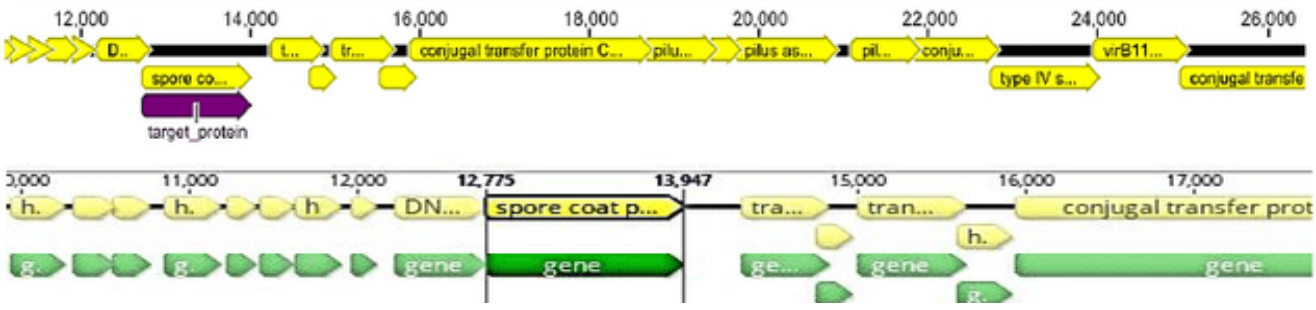

Figure 3. The 42 kDa subunit pili protein sequence of *Salmonella typhi* contains information on the location of the spore coat protein gene (CotH)

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%, Ilu 4.4%, and Leu 6.4% of the AA composition (Table 1). It is expected that Ala, Val, Ilu, 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 hydropathic value ranges from -2 to +2, with the higher (positive) hydropathic 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.

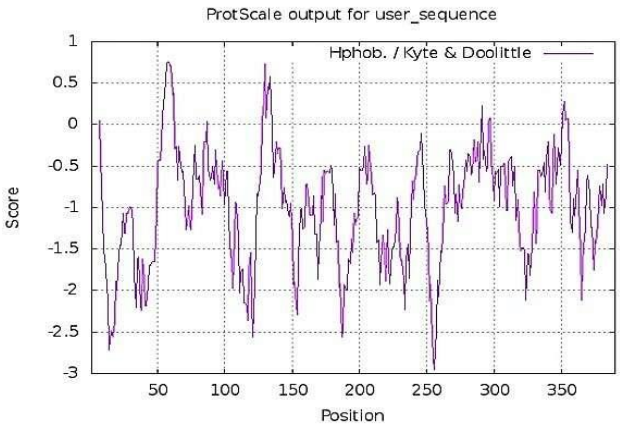

Figure 4. Plot graph of the HpHob./Kyte & Doolitle hydropathy protein of 42 kDa subunit protein *Salmonella typhi*

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*

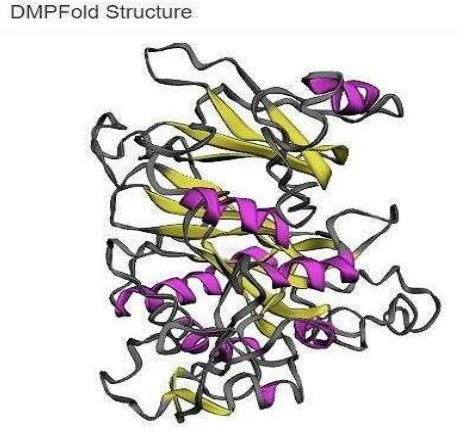

Figure 6. Secondary structure prediction of the 42 kDa subunit protein of *Salmonella typhi*

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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