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**Short Communication**

# Protein Isolation and Purification of Sea Cucumber (*Holothuria* sp. and *Stichopus* sp.) from Sepanjang Beach, Yogyakarta

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**ABSTRACT**
*Holothuria* sp. and *Stichopus* sp. at Sepanjang Beach, Yogyakarta were studied for protein isolation. The crude protein concentrations in the body wall and intestine of *Holothuria* sp. were 2.42% and 2.13%, while *Stichopus* sp. had 4.03% and 4.01%, respectively. Proteins identified through SDS-PAGE analysis included collagen, softenin, and actin. Purified protein concentrations in *Holothuria* sp. were 3.72 ± 0.04 µg mL$^{-1}$ (body wall) and 4.09 ± 0.05 µg mL$^{-1}$ (intestine), while *Stichopus* sp. had 3.98 ± 0.08 µg mL$^{-1}$ and 9.34 ± 0.48 µg mL$^{-1}$, respectively. This study supports nutraceutical development, identifying potential health-enhancing supplements and drugs.



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Sea cucumbers are marine organisms with high economic value due to their nutritional content. Dried sea cucumbers contain 69-82 % protein, 1.7 % fat, 8-8.9 % water or moisture, 4.8 % fibre and 1 % lipid (Maskur et al. 2024; Ibrahim et al. 2015), while fresh sea cucumbers contain 87.78 % moisture, 65.53 % protein, and 1.76 % fat (Fawzya et al. 2015). Sea cucumbers offer a variety of health benefits and serve as a popular source of medicinal ingredients. Furthermore, sea cucumbers have various biological activities such as antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, and neuroprotectant. The protein content in sea cucumbers can be used as a therapy for various diseases (Ovchinnikova 2019; Al-Khayri et al. 2022). Fortunately, Indonesia has abundant sea cucumbers that spread widely across the beaches, especially in Yogyakarta. *Holothuria scabra* and *Stichopus hermanii* are two species commonly used as a health-promoting agent in traditional Asian medicine. Its extract has several health benefits, such as suppressing inflammation, promoting wound healing, and improving immunity (Pranweerapaiboon et al. 2021). Moreover, the abundance, underutilization, and potential of cultivating marine species such as sea cucumbers have led to extensive research into using them as a protein source (Guo et al. 2020). However, there hasn't been much research on protein isolation and purification of *Holothuria* sp. and *Stichopus* sp. from Sepanjang Beach, Yogyakarta.

Amalia et al. (2015) reported the abundance of several sea cucumber species in Sepanjang Beach, Yogyakarta, including *Holothuria* sp., *Stichopus* sp., and *Actinopyga* sp. They are also commonly found in several regions, including Tomini, Gorontalo (Daud et al. 2023; Olii et al. 2024), Raja Ampat, Papua (Handayani et al. 2017), Saparua Island, Central Maluku (Lewerissa 2014), Sulawesi (Hisam et al. 2022), and Nyamuk Island, Central Java (Mustagfirin et al. 2021). Additionally, it is cultivated in Karimun Jawa , Central Java (Widianingsih et al. 2024), Sangihe Islands, Sulawesi (Tarimakase et al. 2020), Southeast Maluku (Tomatala et al. 2020), and Kupang, East Nusa Tenggara (Menge et al. 2023). These species have several compounds that have therapeutic properties such as bioactive peptides, collagens, vitamins, minerals, fatty acids, collagen, gelatine, and amino acids. Protein hydrolysates from sea cucumber also have several benefits for health, such as antioxidant, neuroprotective, antiaging, anti-inflammatory, and anticancer (Pangestuti & Arifin 2018). This has proven that the proteins from sea cucumber have biological activity and has significant potential as a health-promoting agent (Man et al. 2023). Given the health benefits of sea cucumbers, it is essential to investigate their protein properties for potential health applications. This research aimed to isolate and to purify proteins from *Holothuria* sp. and *Stichopus* sp. found on Yogyakarta's beaches using anion exchange chromatography, targeting negatively charged proteins common in marine organisms due to their acidic amino acids (Senadheera et al. 2020). This study lays the groundwork for future research into sea cucumber proteins.

*Holothuria* sp. and *Stichopus* sp. were collected in January 2023 from Sepanjang Beach, Gunungkidul, Yogyakarta, Indonesia, extending from the coastline to the intertidal zone. The fresh weight of the sea cucumbers obtained varies from 100 to 150 grams. The body wall and intestine were pulverised using a grinder that had previously added liquid nitrogen. The sample was subsequently immersed in absolute ethanol (1:2 w $v^{-1}$) for the defatting. This research employed modified protein isolation approach based on Wang et al. (2020). Body wall and intestine samples of *Holothuria* sp. and *Stichopus* sp. were added to the lysis buffer with a ratio of 1:2. Lysis buffer consisted of 100 mM $NH_4HCO_3$, 6 M urea, and 0.2 % SDS, and protease inhibitors were added in a ratio of 1:100 between protease inhibitor and lysis buffer were used. Samples were extracted using ultrasonic-assisted extraction for 5 minutes at a frequency of 300 Hz. After the isolation process, the samples were subjected to freeze-drying.

Protein content was determined using Bradford assay and Bovine Serum lbumin (BSA) as a standard, in accordance with procedure of Samah (2019) with optimisation . Samples were read with spectrophotometer at λ 595 nm. The molecular weight of the protein was confirmed using sodium dodecyl sulphate-polyacrylamide gel-electrophoresis (SDS-PAGE) with gradient gel concentration 4-12 % and Tris-Glycine SDS as a running buffer. This protocol referred to Smith (2011) with minor modifications. Protein purification was conducted using anion exchange chromatography by HiTrap Q HP and referred to factory protocol by GE Healthcare with series number 71-7149-00 AP. The start buffer was prepared using Tris – HCl 0,02 M pH 8, then an elution buffered with Tris – HCl 0,02 M pH 8 combined with NaCl 1 M (1:1 v $v^{-1}$). Gradient buffers were also prepared using NaCl 0.5; 1; 1.5; 2; and 2.5 M, and Tris – HCl from the start buffer was added about 100 µL per 5 mL The eluted protein was quantified with Bradford Assay.

Protein from sea cucumber was soaked in lysis buffer and protease inhibitor and isolated using the sonication method. Sonication, a widely used technique in protein extraction, employs ultrasonic waves to disrupt the cell membrane and facilitates the release of proteins (Kim et al. 2013). The primary function of ultrasound is to create bubble cavitation within the biological material, and the subsequent release of energy as these bubbles burst impacts the cell membranes, leading to their disruption (Kadam et al. 2015).

The sample identification was conducted based on observable morphological characteristics in accordance to identification guidebook by Wirawati et al. (2019). The results indicated that the collected sea cucumber samples are *Holothuria* sp. and *Stichopus* sp. (Supplementary Figure 1), further studies are needed for detailed species identification.

The measurement of protein content referred to bovine serum albumin (BSA) as standard with an R-squared value close to 1(Supplementary Data 1) The protein contained in the freeze-dried powder from *Holothuria* sp. and *Stichopus* sp., as shown in Table 1. The samples were subjected to freeze-drying to remove moisture, thereby stabilising the protein and preventing denaturation and aggregation. Freeze-drying removes water by converting it into vapor through sublimation, reducing the risk of protein instability caused by hydration stress (Chen et al. 2021).

This study represented a preliminary investigation into the isolation and purification of proteins from *Holothuria* sp. and *Stichopus* sp. collected at Sepanjang Beach, Gunungkidul, Yogyakarta. The study focused solely on protein isolation and purification. Further comprehensive research is needed to identify specific protein types and their benefits. The research aimed to determine the protein yield obtained through isolation, to characterise the type of protein based on molecular weight using SDS-PAGE, and to quantify purified proteins through anion exchange chromatography. These processes facilitated the initial identification of proteins from *Holothuria* sp. and *Stichopus* sp. as well as the factors affecting these processes. This information might enhance future research on specific protein types and their potential therapeutic activities.

The results indicated that *Stichopus* sp. possesses a higher protein content, around 4 % (20 µg $mL^{-1}$) in both the body wall and intestine, compared to *Holothuria* sp at 2.4 and 2.1 % in the body wall and intestine, respectively. Some studies have reported that *Stichopus* sp. protein content is 34.33 % (Ridhowati et al. 2018) and 41.3 % (Nguyen et al. 2022). Other study revealed that *Holothuria scabra* has a protein content of 4.96 %, while *Holothuria leucospilota* has a protein content of 10.06 % (Yunita et al. 2017). The results indicated that interspecies variations can influence the protein content in sea cucumbers.

**Table 1**. Crude protein content isolated from *Holothuria* sp. and *Stichopus* sp.

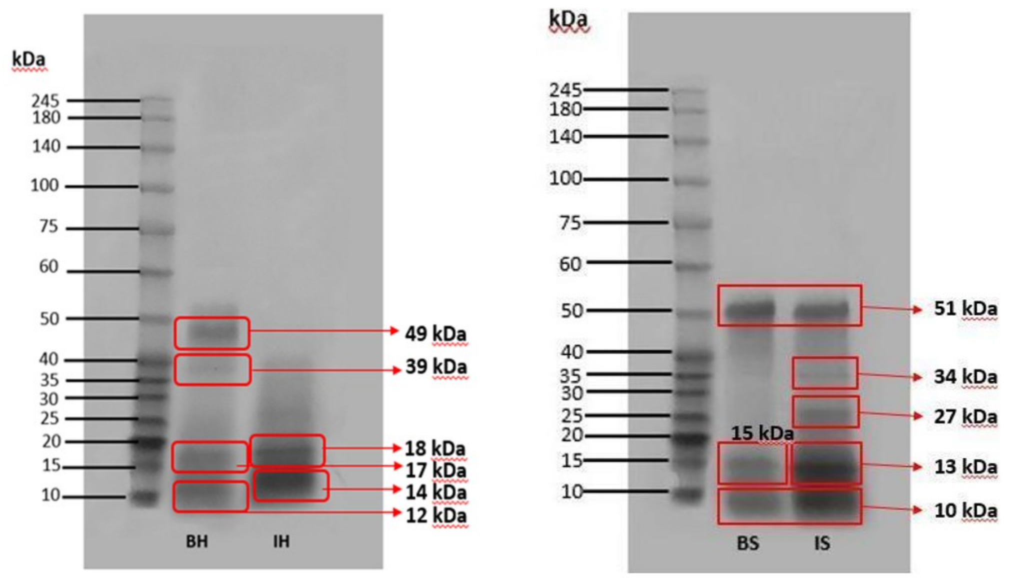
| Sample | Crude sample mass (mg) | Freeze dried protein mass (Mean ± SE, mg) | Freeze dried yield (%) | Protein content (Mean ± SE, µg $mL^{-1}$) |
|---|---|---|---|---|
| BH | 3000 | 46.2 ± 4.47 | 1.54 | 12.11 ± 0.00029 |
| IH | 3000 | 43.31 ± 4.39 | 1.40 | 10.64 ± 0.00032 |
| BS | 3000 | 31.2 ± 4.64 | 1.04 | 20.17 ± 0.00071 |
| IS | 3000 | 39.8 ± 3.52 | 1.32 | 20.06 ± 0.00024 |

Note BH: Body wall *Holothuria* sp., IH: Intestine *Holothuria* sp., BS: Body wall *Stichopus* sp., IS: Intestine *Stichopus* sp.

Additionally, the protein concentration found in this study differed significantly from that reported in some literatures. The differences in protein analysis methods may be the cause of this discrepancy. Apriliani et al. (2024) used proximate analysis to measure protein content and to find the total protein. This study, on the other hand, used an extraction method with a lysis buffer that included 100 mM $NH_4HCO_3$, 6 M urea, 0.2 % SDS, and protease inhibitors to isolate only specific proteins. Therefore, optimising the type of solvent used is necessary to maximise the protein yield. Jafari et al. (2020) stated that protein content and yield during extraction can be influenced by factors such as the solvent and concentration used, sea cucumber species, weight-to-solvent ratio, extraction time, and temperature. Besides that, changes in the conformation of biomolecules in general can cause changes in shape that can adapt to various solvent conditions, namely causing adaptation to the solvent environment, such as changing a polar solvent to a non-polar one. This flexibility additionally affects the function of the compound in vivo. Water is a common solvent in many biological conditions, however molecular stability can also be achieved in non-polar solvents. Protein is a molecule composed of amino acids, half of the amino acids are hydrophobic, namely molecules that avoid contact with water, and also half of the amino acids are hydrophilic, molecules that have direct contact with water. The interaction between the protein and the solvent induces conformational changes in the protein, subsequently affecting its adaptation to the solvent (Meyer et al. 2013; Foumthuim & Giacometti 2023).

In addition to these factors, seasonal variations and food availability can also influence the protein concentration in sea cucumbers. Seasonal changes impact temperature, which in turn affect food source availability and metabolic activity in sea cucumbers, potentially altering protein levels or amino acids content (Feng et al. 2021). Phytoplankton, a food source for sea cucumbers, was found to increase during the winter, so the availability of phytoplankton can influence protein content in sea cucumbers (Deng et al. 2019; Kingsolver et al. 2015). In this study, sampling was conducted in January 2023, during which Sepanjang Beach was still experiencing summer, resulting in limited food availability and affecting the protein content in the sea cucumbers. Torreno et al. (2023) reported that the body wall extracts of *Stihopus horrens* contained elevated levels of structural, diacylated phosphatidylcholines (PCs), suggesting their potential involvement in the synthesis of proteins crucial for the structural integrity and functioning of the sea cucumber's body. Their specific metabolic pathways generate essential bioactive compounds necessary for their survival and environmental adaptation.

The result of SDS-PAGE analysis showed that several proteins were detected in different molecular weights and some patterns are formed between the body walls and the intestine. The results of SDS-PAGE analysis shown in Figure 1. indicated that the *Holothuria* sp. body wall has various protein patterns with molecular weights of around 12, 17, 39, 49 kDa with the thickest band at 12 and 49 kDa. Besides that, intestine *Holothuria* sp. just showed two bands with molecular weights of about 14 and 18 kDa with the thickest band at 14 kDa.




(a) (b)

**Figure 1**. SDS-PAGE pattern of the protein isolated from *Holothuria* sp. (a), *Stichopus* sp. (b). BH: Body wall *Holothuria* sp.; IH: Intestine *Holothuria* sp.; BS: Body wall *Stichopus* sp.; IS: Intestine *Stichopus* sp.

The body wall of *Stichopus* sp. has proteins with molecular weights of around 10, 15, and 51 kDa, while the intestine of *Stichopus* sp. showed proteins of around 10, 13, 27, 34, and 51 kDa. The type of protein with a molecular weight of 12-18 kDa is probably collagen hydrolysate protein. This result aligns with Yusro et al. (2020), who stated that collagen hydrolysates obtained from gold sea cucumber have molecular weight with a range of approximately 14.4 to 25 kDa. Sea cucumbers have a high collagen content because their body walls are predominantly composed of collagen-forming amino acids such as glycine, proline, and hydroxyproline (Senadheera et al. 2020; Gustini et al. 2022). At 27 and 34 kDa, the probably protein detected is softenin. Takehana et al. (2014) indicated that the protein with a molecular weight of around 20-30 kDa is softenin. Softenin is a novel protein that makes the sea cucumber connective tissues softer by inhibiting fibril interactions. At 39, 49, and 51 kDa, the probable protein detected is actin. Truong and Le (2019) reported that the actin from sea cucumbers is detected at 40 - 50 kDa. Actin is the protein that forms most animal muscles and presents significant potential for various biological and biomedical uses due to its versatility and essential role in cellular functions (Haarer et al. 2023; Hatano et al. 2020)

*Stichopus* sp. exhibits a higher protein content than *Holothuria* sp. due to their differences in their metabolic processes. The metabolism of *Stichopus* sp. likely resulted in increased proteins for defense, digestion, and the formation of the outer layer of the sea cucumber (Torreno et al. 2023). In this research, it is showed that the body wall and intestine for both *Holothuria* sp. and *Stichopus* sp. have different patterns. The observed difference pattern may result from varying amino acid compositions that play role in proteins structure (Cuevas-Acuña et al. 2019). Chemical modification including glycosylation or ubiquitination could affect the gel mobility and cause retarded gel results. Other modifications such as phosphorylation, may also influence the mobility of the samples and the formation of bands on SDS-PAGE. Meanwhile, hyperphosphorylation may influence the background or smear observed in the gel (Stanley 2011; Guan et al. 2015; Liu et al. 2015).

After measuring the protein concentration using the Bradford assay, purification was performed using Anion Exchange Chromatography. Anion Exchange Chromatography facilitates protein purification by separating proteins according to their charge differences, achieving high resolution for proteins with equal charges but varying affinities (Kadakeri et al. 2020). Some of the proteins were eluted in low concentrations of salt and some in high salt concentrations. Protein of body wall's *Holothuria* sp. was abundant in 1.5 and 2.5 M NaCl elution gradients with concentrations of 3.72 and 1.03 µg $mL^{-1}$, respectively. In addition, the intestine of *Holothuria* sp. contained high protein concentrations of 4.09 µg $mL^{-1}$ in 0.5 M NaCl and approximately 0.2 µg $mL^{-1}$ in 1.5 M NaCl (Figure 2). Besides that *Stichopus* sp's body wall, proteins were eluted at 0,5 M NaCl with a concentration around 3.98 µg $mL^{-1}$ and 2 M NaCl with a concentration around 3.07 µg $mL^{-1}$. In contrast, for *Stichopus* sp's intestine, proteins were eluted at 0.5 M NaCl with concentration around 1.24 µg $mL^{-1}$ and 1 M NaCl with a concentration around 9.34 µg $mL^{-1}$ (Figure 3).

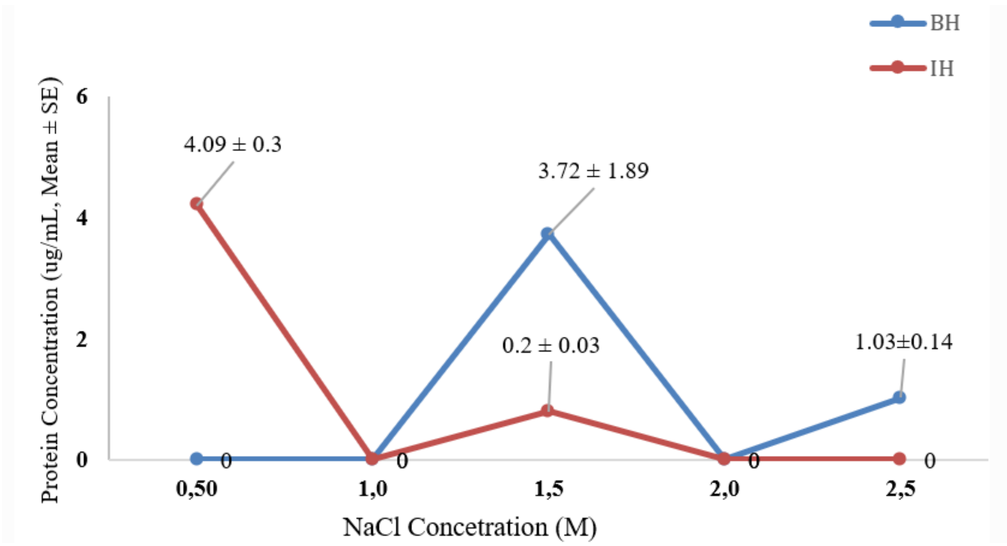



**Figure 2.** Result of Anion Exchange Chromatography from BH: Body wall *Holothuria* sp.; IH: Intestine *Holothuria* sp. The data is presented in the form of Mean ± SE.

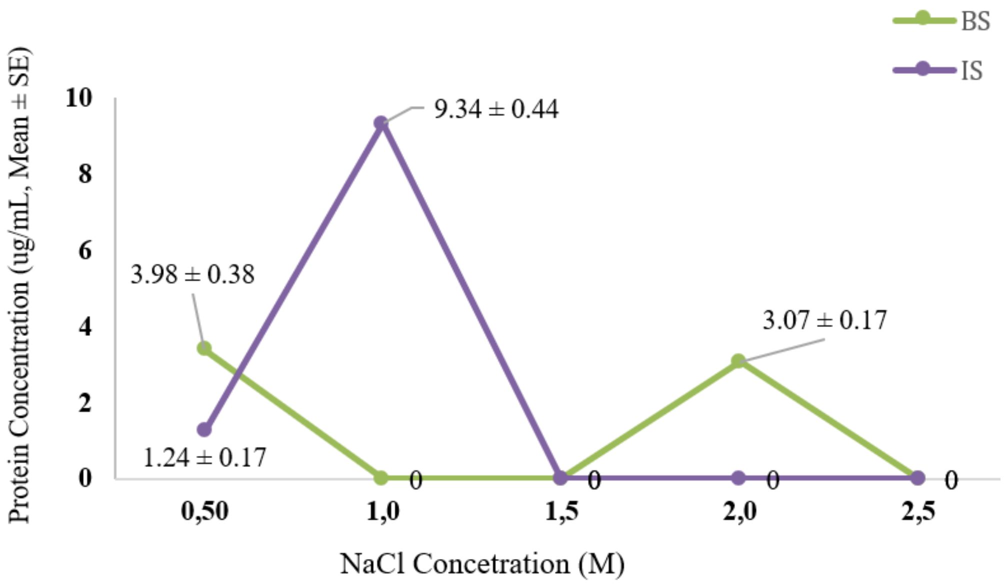



**Figure 3.** Result of the Anion Exchange Chromatography from BS: Body wall *Stichopus* sp.; IS: Intestine *Stichopus* sp. The data is presented in the form of Mean ± SE.

The purified protein of *Holothuria* sp.'s body wall and intestine showed the highest concentration with NaCl elution at 1.5 and 0.5 M. Meanwhile, the body wall and intestine of *Stichopus* sp. showed the highest purified protein concentrations with NaCl elution at 0.5 and 1 M. These results indicated that NaCl 0.5; 1; and 1.5 M can be an effective choice as elution in protein purification of both *Holothuria* sp. and *Stichopus* sp. using Anion Exchange Chromatography. As an alternative, it is necessary to re-optimise with either lower or higher NaCl concentrations to get optimum results. The different concentrations of purified protein from each NaCl elution showed that proteins isolated from both organs have different ionic negativity and could be eluted with different NaCl concentrations. Protein will be eluted by gradient NaCl concentration from low to high, respectively. NaCl with low concentration elute protein with a low ionic charge and NaCl with high concentration elute protein with a higher negative charge (Shire 2015). Salt (NaCl) is used as an eluent in Anion Exchange Chromatography. This NaCl dissociate into $Na^+$ and $Cl^-$ ions in solution, and $Cl^-$ compete with proteins that have a negative charge to bind to the positive stationary phase. Negatively charged proteins that cannot bind due to disruption of $Cl^-$ bonds will dissolve and elute (Acikara 2013).

Proteins from *Holothuria* sp. and *Stichopus* sp. had been extracted. The protein yield in the freeze-dried extract is approximately 1.54 % in the body wall and 1.40 % in the intestine of *Holothuria* sp. *Stichopus* sp. exhibits protein yields of 1.04 % in the body wall and 1.32 % in the intestine. The results of this research indicated that *Stichopus* sp. has a higher protein content, at 4 %, in both the body wall and the intestine, compared to *Holothuria* sp. The most abundant proteins detected by SDS-PAGE analysis were collagen, softenin, and actin. After purification using Anion Exchange Chromatography, the protein concentrations in *Holothuria* sp. are 3.72 µg $mL^{-1}$ in the body wall and 4.09 µg $mL^{-1}$ in the intestine. Concentration purified proteins in *Stichopus* sp. were about 3.98 µg $mL^{-1}$ in the body wall and 9.34 µg $mL^{-1}$ in the intestine. The result of this research needs further confirmation and development to understand the detailed characteristics of sea cucumber proteins, using advanced technologies such as Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS), and to test their activities for various applications.

## AUTHOR CONTRIBUTION

B.K.A., S.L.U., and T.R.N. designed the study. B.K.A. and S.L.U. conducted the laboratory work, analysed the data, and wrote the manuscript. L.H., Z.R., and Y.A.P. were the supervisors. T.R.N. was the supervisor and corresponding author. All authors read and approved the final version of the manuscript.

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## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest to disclose.

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