



Characterization of Trypsin-like Serine Proteases from *Tachypleus tridentatus* Amebocyte Lysate and its Potential for Endotoxin Detection

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ABSTRACT

Background: Serine Proteases in the *Tachypleus* Amebocyte Lysate (TAL) from horseshoe crab, *Tachypleus tridentatus* have many applications in biomedical industries. The sensitivity of these enzymes is a crucial factor for such usages. Until today, the catalytic behavioural and biochemical stability of these enzymes has not been properly addressed. This report aimed to characterize the physicochemical properties of serine proteases to determine their kinetic reactions and to determine the potential utilization of the enzymes for endotoxin detection.

Methods: The purification process of protease from crude TAL was performed using a two-phase filtration unit by affinity and gel columns and the molecular weights of the enzyme fraction from crude, affinity and gel filtration columns were determined. The Azocasein, Aldolase and transaldolase assays were used for the determination of the enzyme Catalysis. The chromogenic assay was carried out to determine the bacterial endotoxin effects using a standard endotoxin from strain *Escherichia coli* 0113: H10 as a positive control. The absorbance was measured at 405 nm. The enzyme activity was determined using the constructed p-nitroanilide standard curve.

Result: A 72 kDa trypsin-like enzyme was purified at fraction-14 (F14) gel filtration and identified as transaldolase. The purified-F14 hydrolysed trypsin substrate, N α -Benzoyl-L-Arginine-p-nitroanilide at higher activity than chymotrypsin. In conclusion, the purified enzyme showed a great potential for enhancing the TAL sensitivity.

Key words: Bacterial endotoxin, Horseshoe crabs, *Tachypleus* amebocyte lysate.

INTRODUCTION

Serine Proteases are an endopeptidase that hydrolyses peptide bonds at the active site of serine residues, a polar amino acid. These enzymes are involved in various physiological and pathological processes such as protein metabolism, digestion, blood coagulation, apoptosis, fertilization and immunity (Carretas-Valdez *et al.*, 2020).

Proteolytic degradation is a major problem that hinders enzyme stability to achieving full potential as catalysts during production, storage and industrial applications. This denaturation often leads to irreversible loss of enzyme activity or inactivation (Sandle, 2016).

Endotoxins are complex lipopolysaccharides (LPS) that form the cell walls of various gram-negative bacteria (Ji *et al.*, 2020). Upon the death of the bacteria, endotoxins are released into the lysate and form bonds with biomolecules, including target therapeutic compounds (Schneier *et al.*, 2020).

Tachypleus amebocyte lysate (TAL) and limulus amebocyte lysate (LAL) screening tests for bacterial endotoxin are widely used in biomedical and healthcare industries. They can analyse a large number of samples and can detect lower endotoxin levels. The assays act by identifying the presence of the Lipopolysaccharide and activate a series of intracellular coagulation pathways leading to the production of a coagulin. The formation of coagulin fibrils is subsequently stabilized by the enzyme transglutaminase and cross-linked with stablin and proxin (Mizumasa *et al.*, 2017).

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The defence mechanisms of Horseshoe crabs are mainly based on innate immune responses which include hemolymph coagulation, phenoloxidase activation, cell agglutination, the release of antibacterial substances, active oxygen formation and phagocytosis. The granular hemocytes in the horseshoe crabs are involved in the storage and release of a variety of defence molecules, including serine protease zymogens and protease inhibitors (Wang *et al.*, 2021). It is crucial to understand the enzyme stability characterization, mechanism of inactivations and

reactions to enable better control over the deactivation process, stabilization approaches and catalytic properties. To our knowledge, the mechanism of enzyme deactivation for thermodynamic stability, substrate specificity and catalytic properties of serine protease contained in the TAL of *Tachypleus tridentatus* remain mostly unknown. This report aimed to characterize the physicochemical properties of serine proteases to determine their kinetic reactions and to determine the enzymes' potential utilization for endotoxin detection.

MATERIALS AND METHODS

Preparation of tachypleus amebocyte lysate (TAL)

Haemolymph of *T. tridentatus* was extracted under sterile condition to obtain amebocyte lysate cells following a patented document (Noraznawati *et al.*, 2014). The duration of the study was 3 years (2017-2020).

The purification process of protease from crude TAL was performed using two-phase filtration units by affinity and gel columns. The lyophilized TAL was reconstituted in 2.5 ml of 0.05 M Tris-HCl buffer at pH 7.4 containing 0.5 M sodium chloride. Then, 10 ml of TAL was inserted into affinity column (Sartorius, Germany), before it was further being injected to a second-phase of filtration through Fast Performance Liquid Chromatography ÄKTA Purifier (GE Healthcare, United Kingdom). The fraction-14 (F14) containing the targeted-protease was then subjected to a second purification step using Hi-Prep 26/60 Sephacryl Gel Filtration Column (Sartorius, Germany). The purification system was performed using 0.02 M Tris-HCl at pH 8 containing 0.1 M sodium chloride flows at a rate of 0.3 ml/min and the protein of the purified-fraction was quantified.

Protein-sequencing of purified protease

The molecular weights of the enzyme fraction from crude, affinity and gel filtration columns were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. For identification of peptide sequence, protein bands from the respective samples of crude, affinity and gel filtration columns were sent to 1st BASE Malaysia for LC-MS analysis. Thereafter, the relative abundance of peptide sequence was identified using Mascot sequence matching software based on Ludwig NR database.

Determination of substrates specificity on enzyme catalysis

The azocasein assay was performed following method described by Tomarelli *et al.* (1949). Firstly, a 2.5% azocasein (Sigma-Aldrich, USA) was reconstituted with 0.5% (w/v) sodium bicarbonate buffer at pH 8.3. The homogenous solution of azocasein (250 µl) was added into a set of sample and blank. Then, sodium bicarbonate was pipetted into each set of sample and blank at a volume of 150 µl and 250 µl, respectively. In a subsequent step, the homogenous mixture of buffer and substrate were incubated at 37°C for 5 min using a water bath (Mettler, Germany). Separately, 100 µl of trypsin (positive-control), sample F14 and blank were

prepared in the new microcentrifuge tubes, followed by incubation at 37°C for 30 min. Next, 400 µl trichloroacetic acid (Merck, USA) was added into the respective microcentrifuge tubes containing trypsin, sample F14 and blank. After incubation (10 min), all set of sample and blank were centrifuged at 8,000 × g for 5 min at 4°C and then 100 µl supernatant from each set of samples and blank were transferred into a new microcentrifuge tubes containing 300 µl sodium hydroxide (Sigma-Aldrich, USA) at a molarity of 0.5 M. After homogenous mixing, 250 µl from each set of sample and blank was loaded into 96-well microtitre plate and the absorbance was measured at 440 nm wavelength. For determination of enzyme activity using azocasein as substrate, the following formula was used as previously adapted by Buarque *et al.* (2009):

$$\text{Enzyme activity (U/ml/min)} = \frac{A_{440\text{nm}} \text{ Test} - A_{440\text{nm}} \text{ Blank}}{0.001}$$

The aldolase assay was performed following method described by Bergmeyer (1974). Five reagents (reagent A, B, C, D and E) were purchased from Sigma-Aldrich (USA). Each reagent was prepared separately consisting of reagent A (0.1 M Tris-HCl at pH 7.4), reagent B (58 mM fructose-1, 6-diphosphate), reagent C (4 mM β-nicotinamide adenine dinucleotide), reagent D (50 unit/ml α-glycerophosphate dehydrogenase) and reagent E (aldolase) at a concentration ranging from 0.25 to 0.50 unit/ml. First, reagent A, B, C and D was added into each microcentrifuge tube and incubated at 25°C using a waterbath (Mettler, Germany). Then, test materials including aldolase (positive control), sample of purified-F14 and reagent A (blank) were added. These samples were inverted, transferred into 96-wells microtitre plate in triplicates and the absorbance was measured at 340 nm wavelength for every 5 min interval at a total duration of 30 min. The enzyme activity was calculated using the following formula:

Enzyme activity (U/ml enzyme) =

$$\frac{(\Delta A_{340\text{nm}} / \text{min test} - (\Delta A_{340\text{nm}} / \text{min blank}) (3) (df))}{(2) (6.22) (0.1)}$$

The transaldolase assay was performed according to Bergmeyer (1974). Seven reagents were prepared consisting of reagent A: 0.25 M glycyl-glycine buffer (pH 7.7), reagent B: 0.1 M D-erythrose-4-phosphate solution, reagent C: 0.2 M D-fructose-6-phosphate solution, reagent D: 0.3 M magnesium chloride solution, reagent E: 2.6 mM β-nicotinamide adenine dinucleotide, reagent F: α-glycerophosphate dehydrogenase or triosephosphate isomerase enzyme solution and reagent G: transaldolase enzyme solution. The assay was performed in triplicates following the steps described earlier. The enzyme activity was calculated using the following formula adapted from Bergmeyer (1974):

Enzyme activity (U/ml enzyme) =

$$(\Delta A_{340\text{nm}} / \text{min test} - (\Delta A_{340\text{nm}} / \text{min blank}) (3) (df))$$

The optimal temperature and enzyme stability for azocasein assay was determined by applying temperature ranging from 30 to 70°C. Test set for enzyme and blank was performed in triplicates using 0.05 M glycine buffer at pH 11. For pH determination, azocasein assay was used as a standard using pH ranging from 7 to 13. Three different buffer formulations with different pH were used including 0.05 M Tris-HCl at pH 7-9, 0.05 M glycine at pH 10-11 and 0.05 mM potassium chloride at pH 12-13 (Haddar *et al.*, 2010; Rao *et al.*, 2009). For measurement of enzyme inhibition, azocasein was used with a slight modification following method described by Rao *et al.* (2009). The inhibition assay was performed by incubating a crude enzyme with 5 mM of enzyme inhibitors phenylmethane sulfonyl fluoride (PMSF), leupeptin, antipain and chemostatin at 30°C for 30 min. The V_{max} and K_m values were determined from Lineweaver Burk plot constructed.

Measurement of tryptic and chemotryptic activity

To construct a standard curve for tryptic and chemotryptic activity, a series of p-nitroanilide (Sigma-Aldrich, USA) at a concentration of 1, 2, 5, 10, 15 and 20 nmol were dissolved in 0.1 M Tris-HCl (pH 7.8). A 50 μ l (40 μ l of substrates and 10 μ l of the test sample) was added to initiate the reaction and incubated at 30°C for 60 min. Then absorbance was read at 405 nm wavelength.

For the endotoxin test, the chromogenic assay was used. For a positive-control, standard endotoxin from strain *Escherichia coli* 0113: H10 (Lonza, USA) at a concentration of

0.5 EU/ml was added to the LAL pyrochrome (Cape-Cod, USA). On the other hand, a mixture of LAL pyrochrome and pyrogen free-water was assigned as a negative-control. In addition, a treatment group comprises a mixture of sample F14 dissolved in pyrochrome chromogenic reagent and stimulated with endotoxin. The absorbance was measured at 405 nm wavelength and the enzyme activity was determined using the p-nitroanilide standard curve constructed.

RESULTS AND DISCUSSION

TAL was purified using two types of column filtration units: affinity and gel filtration columns. The yield of the purified enzyme was about 7.76% of the total enzyme in TAL for the first purification. After the second purification, the pure target enzyme was about 2.33%. The purity of the enzyme increased as demonstrated by specific enzyme activity from 0.86 to 2.38 units/mg. Proteins in the crude TAL, Fraction-1 (F1) derived from the Benzamidine column and Fraction-14 (F14) derived from the gel column showed a reduced trend at concentrations of 2.38, 1.42 and 0.07 mg/ml (Fig 1).

The loss of the targeted purified enzyme might occur through the purification but it was not too much as the procedure was conducted in a cold environment at a temperature ranging from 4 to 8°C. Using SDS-Page profiling, we found that the purified F14 has a molecular weight of 72 kDa. It was identified as two protein fragments namely transaldolase (NCBI accession: I1FU18) and uncharacterized protein (NCBI accession: I1FU18) (Fig 1). Both the proteins showed similar sequences

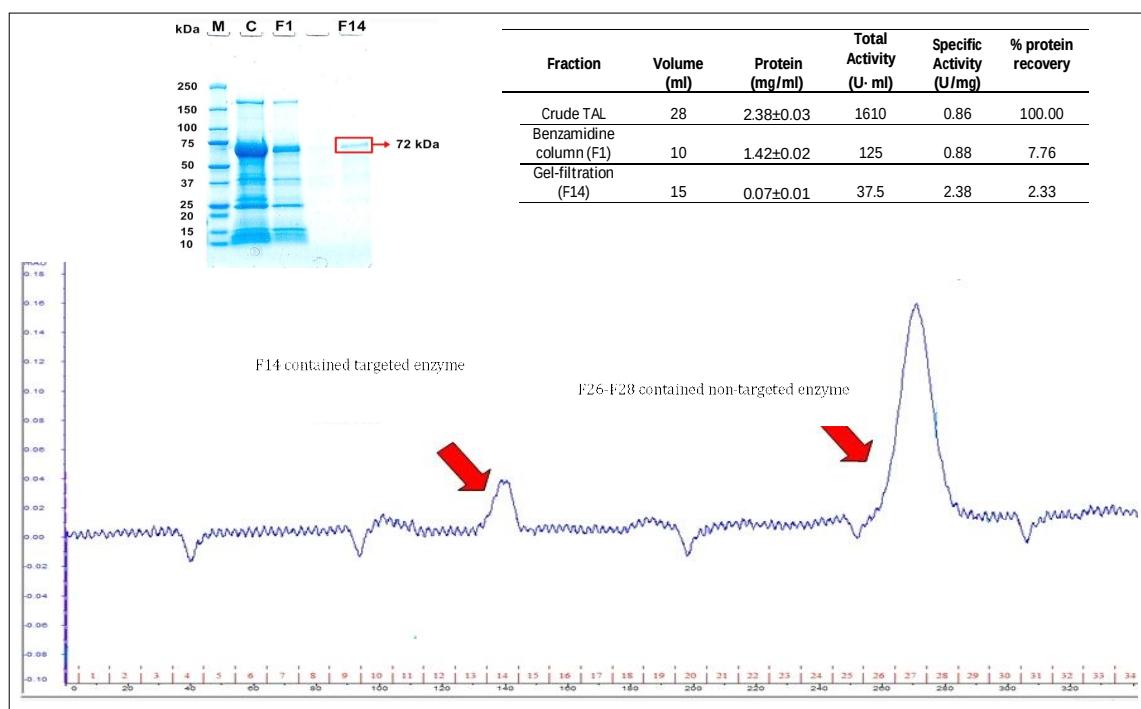


Fig 1: SDS-PAGE protein profiling collected at different stages of purification process using gradient gel at concentration of 4-20% pre-cast (Bio-rad, U.S.). Abbreviation letter indicates M: Marker, C: Crude sample, F1: Fraction-1 collected from Benzamidine column, F14: Fraction-14 collected from gel filtration column.

with *Amphimedon queenslandica* using Mascot, Matrix Science search engine (Table 1).

The enzyme F14 was able to hydrolyse both 3.5 mM N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAPNA) and 4 mM N-Benzoyl-L-Arginine 4-Nitroanilide Hydrochloride (BAPNA) (Fig 2). This indicated that this enzyme possessed both tryptic and chymotryptic characteristics (Muharsini *et al.*, 2000). However, the tryptic activity appeared to be higher as compared to the chemotryptic. This indicates that it was most likely to have trypsin-like characteristics (Mohamed *et al.*, 2005). Both tryptic and chemotryptic activities reached the highest activity during 15 minutes intervals. After 15 minutes, the enzyme activity was consistently dropped, as the bonds between enzyme and substrate were detached at a constant rate due to the lack of substrate to act upon (Price and Steven, 2011). No reaction was observed by the aldolase and the transaldolase assays (Fig 3). Therefore, we concluded that the uncharacterized protein was belonging to the F14 enzyme.

Treatment with various inhibitors shows that the highest inhibition was shown by PMSF, Antipapain, Leupeptin and

Chymostatin at 91%, 87%, 84% and 40% inhibition percentage, respectively (Fig 4a). The observed inhibition effect revealed that the F14 enzyme was serine protease as these inhibitors specifically react towards the serine protease group of the enzyme (Kumar, 2002). The highest percentage of inhibition observed by the PMSF indicated that the enzyme could be identified as serine protease or endopeptidase (Rao *et al.*, 2009; Khan *et al.*, 2007).

The enzyme F14 was found to be active at temperatures ranging from 30°C to 40°C (Fig 4b), then gradually decreased. The reduction of enzymatic activity after 40°C was due to the inactivation from its native state (Rao *et al.*, 2009). The huge degradation effect was observed at 70°C as the protein lost its original structure due to temperature increment. The increment in temperature would increase the energy of molecules which became the major block of protein structure building. In addition, it also increases the chance of the breakdown of weak non-covalent bonds such as the Van Der Waals force (Ceichanover, 2012; Price and Steven, 2011; Hames and Hooper, 2006). The enzyme F14 functions optimally at pH10 (Fig 4c). This finding was in

Table 1: Identification of protein sequence for purified sample of F14 from *Tachypleus tridentatus* amoebocyte lysate using mascot LC-MS/MS analysis.

Estimated molecular weight (kDa)	Protein name	NCBI accession	Species	Ion score	Peptide sequence
36.038	Transaldolase	I1FU18	<i>Amphimedon queenslandica</i>	59	Istevdar
15.302	Uncharacterized protein	I1FU19			

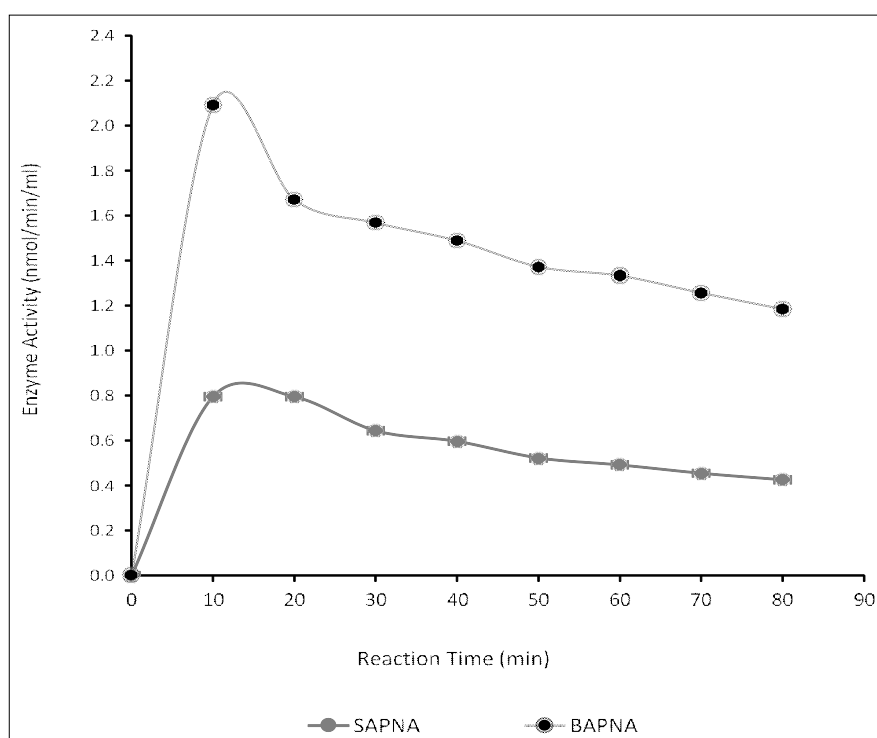


Fig 2: Enzyme activity of sample F14 at different reaction time using 3.5 mM N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAPNA) and 4 mM N-Benzoyl-L-Arg-p-nitroanilide (BAPNA) as chromogenic substrates.

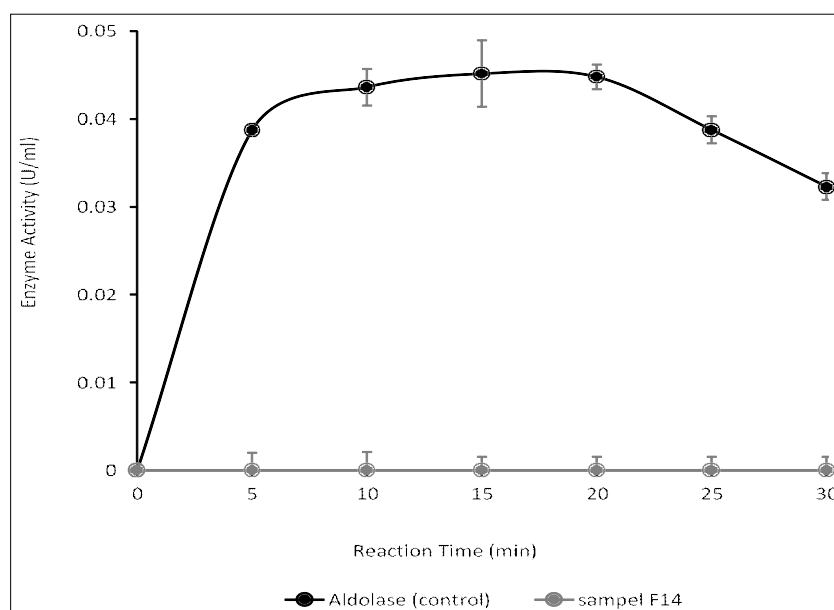


Fig 3: Enzyme activity of F14 sample using aldolase assay.

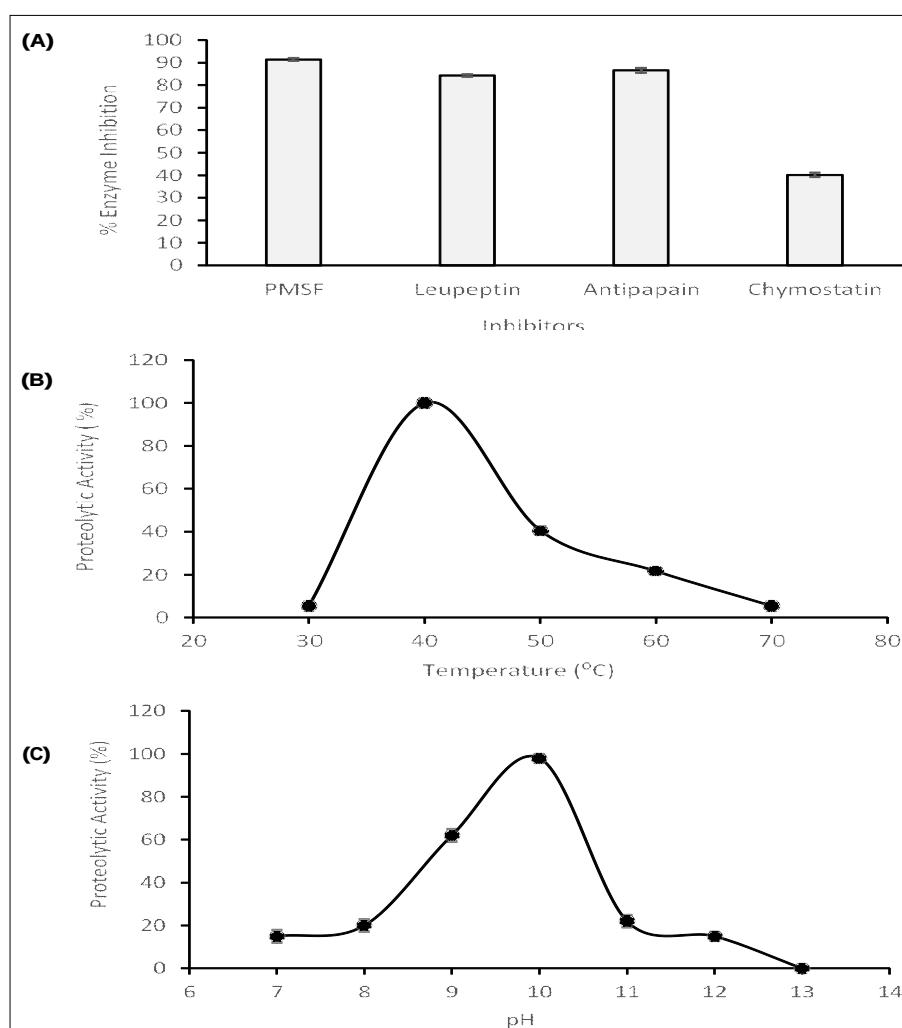


Fig 4: Effect of various inhibitors (A), temperature (B) and pH (C) on percentage of enzyme inhibition of F14 sample using azocasein assay.

agreement with Ghorbel *et al.* (2003), who found that alkaline protease enzymes work best at pH9 to pH10. Besides, it also confirmed that the F14 has a similar characteristic of trypsin which was reported to react within a range of pH7 to pH 10.5 (Muhlía-Almazán *et al.*, 2008). The maximum velocity, V_{max} and Michaelis-Menten constant, K_m for the F14 enzyme's reaction in the azocasein reaction were determined as 0.34 U.s⁻¹ and 1 mM respectively (Fig 5). These results indicated that at least 0.34 μ mol per second of products were produced. The maximum velocity, K_m value

which was as low as 1 mM (9.97×10^{-4} M) revealed that the enzyme possessed a very high affinity towards substrates (Hames and Hooper, 2006).

After the addition of the F14 enzyme in the assay, a significantly higher rate of detection was observed at 5, 10 and 15 min of intervals (Fig 6). After 15 min reaction, most of the substrate was converted into a product which in turn dropped the enzyme activity constantly (Bonner, 2007). At 10 min reaction time, the enzyme activity of the treatment group with the addition of F14 enzyme was significantly

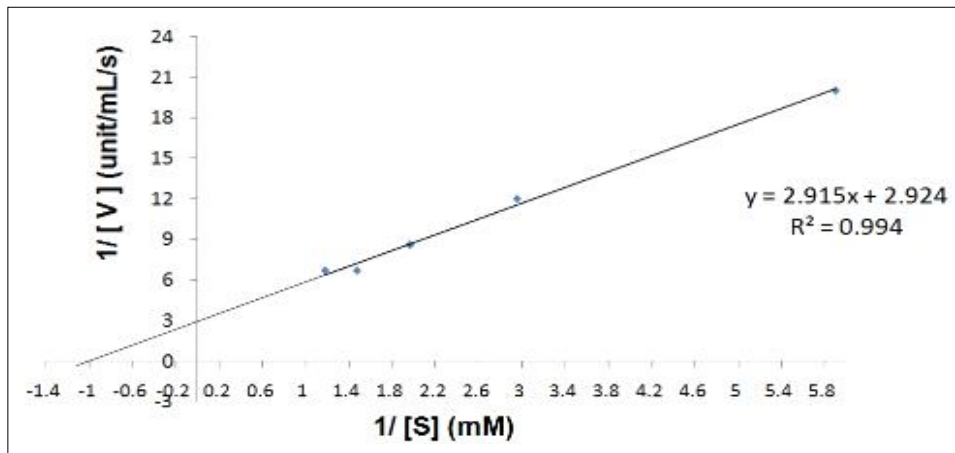


Fig 5 : Determination of maximum velocity, V_{max} and Michaelis-Menten constant, K_m for F14 enzyme's reaction in the azocasein reaction.

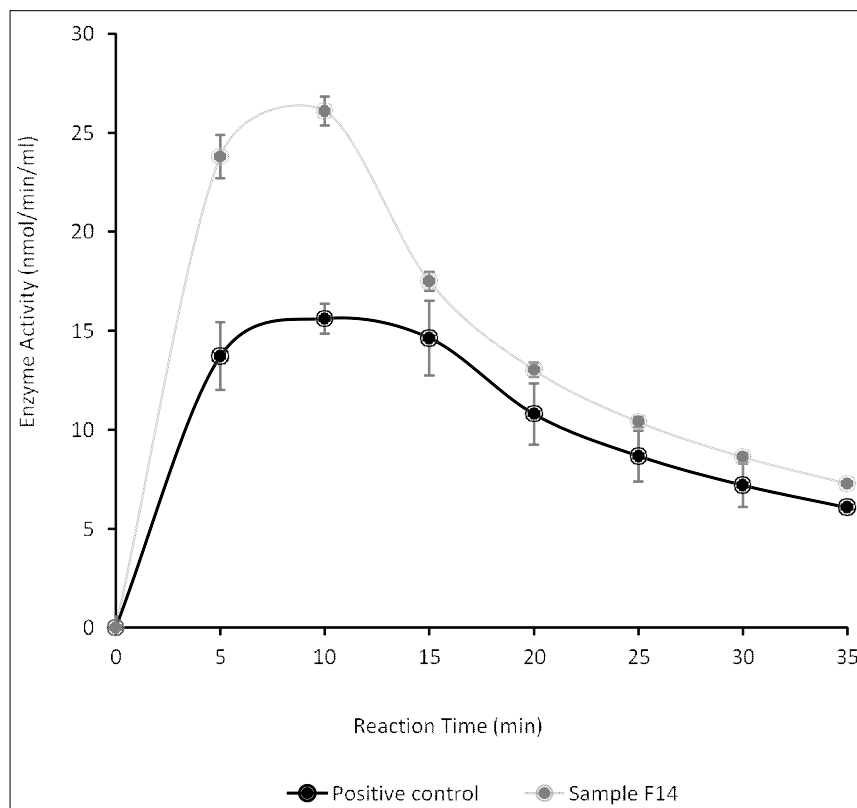


Fig 6: Enzyme potency of F14 sample to strain *Escherichia coli* 0113: H10 at a concentration of 0.5 EU/ml using chromogenic test.

($P < 0.05$) higher (26.10 nmol/min/ml) as compared to the control group (15.61 nmol/min/ml) of the product yield. These findings suggested that the addition of the F14 enzyme could shorten the reaction time for the bacterial endotoxin test.

CONCLUSION

The isolated enzyme (F14) in this study was identified as a trypsin-like serine protease. The F14 enzyme was able to increase the reaction rate for endotoxin detection and; therefore, increase the sensitivity of TAL. The outcome of this study supports the industrial utilization of enzymes for endotoxin detection and as a potential biosensor.

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Conflict of interest: None.

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