

## Isolation and purification of angiotensin converting enzyme inhibitory peptide from goat milk hydrolysate by ultrafiltration membrane

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### Abstract

Hypertension been a significant risk factor for cardiovascular illness. Synthesized angiotensin converting enzyme (ACE) inhibitors are active antihypertensive medicines they frequently generate unwanted side effects. This study describes the isolation and purification of an ACE inhibitory peptide from goat milk hydrolysate (Sahel breed). Hydrolysates containing angiotensin converting enzyme (ACE) inhibitory peptide were produced from goat milk by pepsin and trypsin. The strongest ACE inhibitory action was found in pepsin hydrolysate (57%) at 12h, hence was further purified. Purification was done using a 10 kDa ultrafiltration membrane and three steps of RP-HPLC to obtain ACE inhibitory peptide with activity of 84% and IC<sub>50</sub> of 3.25±0.11 µg/ml. Lineweaver–Burks' plots showed that the inhibitory kinetic mechanism of this peptide was competitive. In conclusion, ACE inhibitory peptide from goat milk hydrolysate treated with pepsin may be positive constituent to serve as drug against hypertension.

**Key words:** Hypertension; ACE Inhibitory Peptide; Goat Milk Hydrolysate; Purification.

### 1. Introduction

According to [1] and [2], Worldwide, hypertension affects 15-20% of adults and is the primary cause of cardiovascular illness., accounting for 7.5 million deaths annually. It is also a major public health concern in both developed and developing nations. By 2025, it is expected that 1.56 billion individuals would have hypertension, despite improvements in treatment options [3]. Cardiovascular disease can harm a variety of target organs at varying rates, including the kidney, brain, and eye [4]. It affects the structures and operations of small muscle arteries, arterioles, and other blood vessels. As people get older, the likelihood of developing hypertension rises.

In the first-line of treatment for hypertension, ACE inhibitor is used. The peptidyl peptidase hydrolase ACE is crucial for the physiological control of hypertension. Angiotensin I, an inert decapeptide, is converted into the active vasoconstricting octapeptide angiotensin II [5]. Captopril, ramipril, lisinopril, enalapril, and other popular ACE inhibitors are currently on the market, however they all have unfavorable side effects. As a result, interest has grown in looking for all-natural remedies that could reduce hypertension [6].

Milk, soy proteins, fish proteins, and other proteins have been found to include peptides with ACE inhibitory action [7]. Some of these bioactive peptides, according to [8], have anti-oxidative, anti-carcinogenic, antibacterial,

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immunomodulatory, antithrombotic, mineral binding, and ACE inhibitory properties. As a result, the goal of this work is to extract and purify ACE inhibitory peptides from goat milk (Sahel breed).

## 2. Materials and methods

Angiotensin converting enzyme from rabbit lungs, Hippuryl-histidyl-leucine (HHL), pepsin and trypsin (Sigma-Aldrich, St. Louis, MO 63103, USA). All other reagents used in the study were of analytical grade.

### 2.1. Equipment's

Refrigerated centrifuge (MSB005.CR2.K, MSE Ltd, U.K), HPLC Machine (Adept CECIL CE4201) and spectrophotometer (JENWAY 6300) (Bibby scientific limited, UK), Spectrophotometer CE7400 (CECIL, Cambridge England), Oven (OV/100), Incubator (MINI/50/VIS) and pH meter (Genlab, Metrohm Ltd., Herisan, Switzerland), Electrical weighing balance and Freeze dryer (LabogeneApS, Denmark).

### 2.2. Collection and Preparation of Milk Sample

Raw unpasteurized milk was collected from five (5) goats (Sahel breed) in Tudun Wada Local Government Area, Gombe State, Nigeria. The milk was combined and transported to the laboratory while on ice. The milk was centrifuged for 15 minutes at 4°C and 4000xg to remove the fat, and the supernatant was transferred to a fresh container. This was centrifuged again to ensure that as much fat as possible was removed, leaving only the skimmed milk [9].

### 2.3. Preparation of Hydrolysates

The hydrolysate was prepared in accordance with [10] instructions. The pH of the skimmed milk was adjusted to 8.0 and 2.0 using 0.5 N NaOH and 0.5 N HCl, respectively. The pH of the reaction mixture was maintained by adding trypsin and pepsin used for hydrolysis at a proportion of 1:100 (enzyme:substrate, w/w, protein basis) to skimmed milk (pH 8.0 and 2.0 for trypsin and pepsin, respectively). After 6, 12, 18, and 24 hours of hydrolysis, samples were extracted and the enzymes were inactivated in a 90°C water bath for 10 minutes. The hydrolysates were centrifuged (12,000 g), and the resultant supernatant was tested for ACE inhibitory action.

### 2.4. Determination of Protein Content

Protein concentration was determined using Biuret assay method with Bovine Serum Albumin (BSA) as standard and the concentration was expressed in milligram per milliliter (mg/ml) [11].

### 2.5. Determination of ACE Inhibitory Activity

The ACE inhibitory activity was measured using a modified [12]. Hippuryl Histidyl Leucine liquified in a 50 mM Na<sub>3</sub>Bo<sub>3</sub> buffer comprising 1N NaCl. The mixture of 25 µl of 5 mM Hippuryl Histidyl Leucine solution and ten liter of milk hydrolysate was then pre-incubated for 10 minutes at 37 °C. The activity was started through adding 10 µl of Angiotensin Converting Enzyme, the combination was incubated at 37 °C for thirty minutes. The reaction was halted via addition of 200 µl of 1N NaCl. After removing the ethyl acetate by vacuum evaporation, the released hippuric acid was removed with 1 ml ethyl acetate and liquified through addition of the buffer (1 ml). The OD at 228 nm was measured, and rate of inhibition was determined using the following.

$$\text{ACE Inhibition (\%)} = (B - A) \div (B - C) \times 100$$

A denotes the OD in the presence of Angiotensin Converting Enzyme with an ACE-inhibitory component, B denotes the OD in the absence of an ACE-inhibitory component, and C is the OD in the absence of ACE.

The hydrolysate/peptide fraction's inhibitory activity was expressed as a percentage of ACE inhibition at a specified protein concentration or as per the concentration required to inhibit 50% of the original ACE activity (IC<sub>50</sub>).

### 2.6. Purification of ace inhibitory peptides from hydrolysates

#### 2.6.1. Protein Ultrafiltration

The hydrolysate (supernatant) obtained from hydrolysis of the goat milk was filtered through an ultra-filter (Sigma-Aldrich, USA) using millipore express® PLUS PES, a polyethersulfone (PES) membrane to remove molecules larger than 10,000 kDa. The hydrolysates' protein content and ACE inhibitory activity were then assessed.

### 2.6.2. Reverse Phase Liquid Chromatography (RP-HPLC)

The hydrolysate after ultrafiltration was further purified by a three-step reverse phase HPLC. One milliliter of the hydrolysate was put to a reverse-phase column and eluted at a flow rate of 1 ml/min in a linear gradient mode of acetonitrile (0 to 40% for 40min, 0 to 30% for 20min, 0 to 20% for 30min) containing 0.1% trifluoroacetic acid. At 215 nm, the elution was measured, and fractions were collected for ACE-inhibitory tests. The fraction with the strongest ACE-inhibitory activity was then collected and analyzed further.

### 2.6.3. Determination of the ACE Inhibitory Pattern of the Purified Peptide

The ACE inhibition pattern was assessed using methods modified from [13] and [14]. ACE inhibition was determined by co-incubating different concentrations of substrate (HHL) (0.1, 1, 2, and 4 mM) in the absence and presence of 0.8 and 1.2mg/ml of partially purified peptide. Lineweaver-Burk plots were used to calculate the kinetic constants ( $K_m$  and  $V_{max}$ ) for the process at various peptide concentrations.

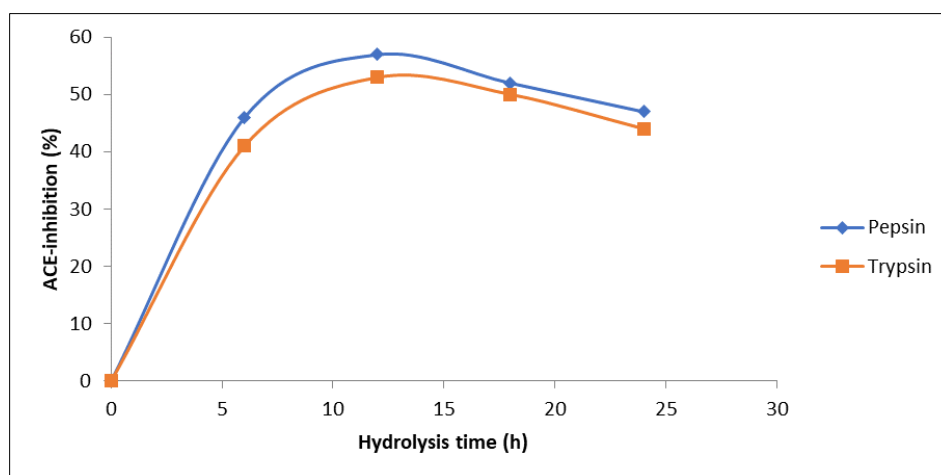
## 2.7. Statistical Analysis

Data were expressed as mean of three replicates  $\pm$  standard deviation (SD). The data were subjected to analysis of variance using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

## 3. Results and Discussions

### 3.1. Purification of ACE Inhibitory Peptide(s) from Hydrolyzed Goat Milk

Enzymatic hydrolysis is chosen over microbial fermentation for the generation of bioactive peptides due to its short response time, easy-to-scale-up, and predictability. To hydrolyze proteins, more than one proteolytic enzyme (purified or crude) can be used, resulting in hydrolysates having short peptide sequences [15]. From results of the present study (Figure 1), ACE inhibition reached its peak after 12hours of hydrolysis and then gradually reduced. [16] observed a comparable drop in ACE inhibiting peptides derived from lizardfish (*Synodus macrops*) scale gelatin, concluded that the decline was most likely due to the release of the most potent Inhibitory peptides for ACE at the onset of hydrolysis. The high Inhibitory action of ACE by pepsin hydrolysate (57%) at 12hours compared to trypsin (53%) in the present investigation is consistent with previously published results demonstrating pepsin hydrolysates demonstrate stronger ACE inhibitory activity compared to hydrolysates of other enzymes [17].



**Figure 1** ACE inhibitory activities of peptides produced by enzymatic hydrolysis of goat milk (Sahel breed) using pepsin and trypsin

Table 1 summarizes the purification steps for the ACE inhibitory peptide isolated. The amount of crude protein was 35.42 mg/ml with a specific inhibitory activity of 0.0042 U/mg. After ultrafiltration, the specific inhibitory activity increased to 0.0089 U/mg at a recovery rate of 71% and purification fold of 2.12. The specific inhibitory activity of the peptide further increased to 0.0308 U/mg, 0.0812 U/mg and 0.1548 U/mg after purification with three successive RP-HPLC steps (Table 1). Purification of ACE inhibitory peptides from various sources has been accomplished using ultrafiltration, gel filtration, ion exchange chromatography, and HPLC [18,19]. In this study, ultrafiltration and RP-HPLC were employed to obtain the purified peptide. Although column chromatography can be used to obtain purified protein

fractions from appropriate columns, ultrafiltration is a cost effective with ease of scalability technology frequently utilized in the food industry for fast separation and concentration of dietary proteins [20] and has been successfully used by several researchers to isolate ACE inhibitory peptides [21].

**Table 1** Purification Profile of ACE Inhibitory Peptide from Goat Milk Hydrolysate

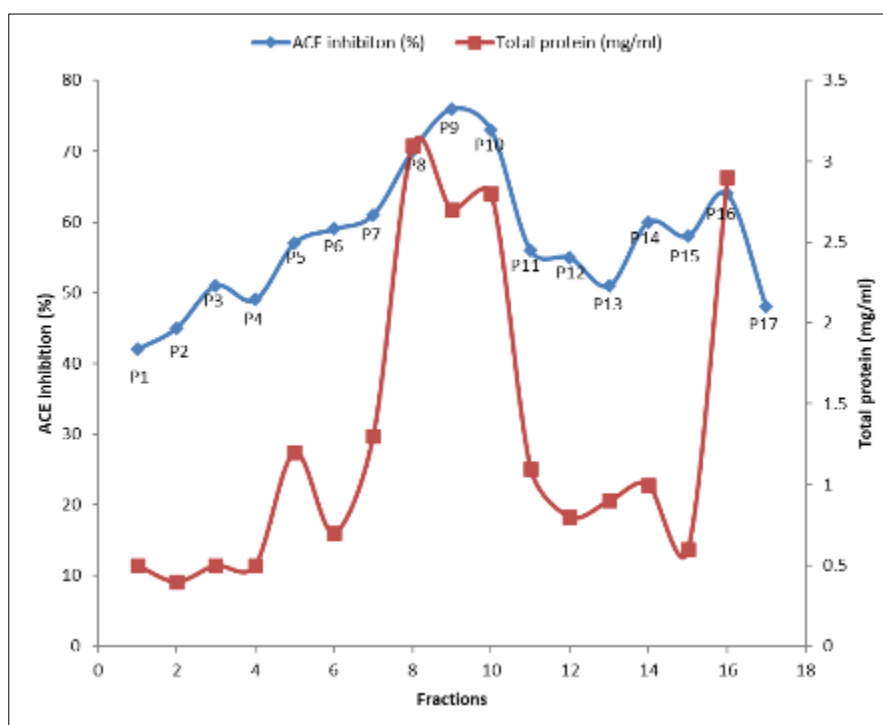
Purification steps	Protein Content (mg/ml)	Total Protein (mg)	Inhibitory Activity ( $\mu\text{mol}/\text{min}/\text{ml}$ )	Specific Inhibitory Activity (U/mg)	Purification Fold	Yield (%)
Crude	11.80	35.42	0.1504	0.0042	1	100
Ultrafiltration	4.04	12.11	0.1075	0.0089	2.12	71
1 <sup>st</sup> RP-HPLC	0.92	2.76	0.0849	0.0308	7.33	56
2 <sup>nd</sup> RP-HPLC	0.32	0.95	0.0775	0.0816	19.43	52
3 <sup>rd</sup> RP-HPLC	0.14	0.42	0.0650	0.1548	36.86	43

1U of inhibitory activity was defined as the quantity of inhibitor that reduced ACE activity by 50%.

### 3.2. Purification of ace inhibitory peptides from hydrolysates

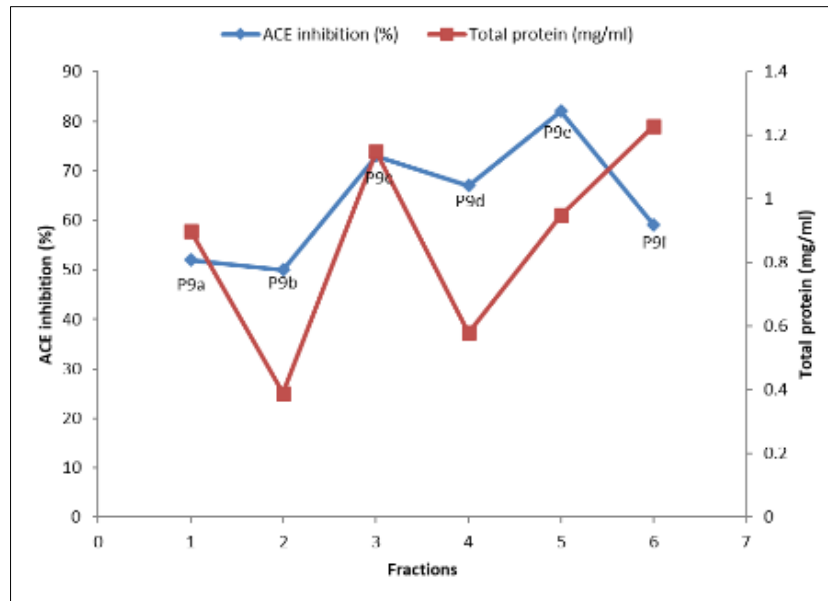
Figure 2 shows the ACE inhibitory fraction's profile from the first RP-HPLC purification step. Seventeen (17) fractions designated as P1 to P17 were obtained and the ACE inhibitory activity as well as total protein content of each fraction were assayed (Figure 2). Fractions P1, P2, P4 and P17 did not exhibit inhibitory activity, while there was activity from fraction P5 to P16. The fraction with the peak ACE-inhibitory activity was P9 (76%) with  $\text{IC}_{50}$  of  $4.24 \pm 0.07 \mu\text{g}/\text{ml}$  and total protein of 2.7mg while the lowest activity was observed in P1 (42%).

After the second purification step by RP-HPLC, six (6) fractions (from P9a to P9f) were obtained (Figure 3). The elution profile (Figure 3) shows inhibitory activities of 52%, 50% and 59% for fractions P9a, P9b and P9f respectively, while inhibitory activities of fractions P9c, P9d and P9e increased, with fraction P9e having the strongest inhibitory activity against ACE (82%) having  $\text{IC}_{50}$  of  $3.87 \pm 0.33 \mu\text{g}/\text{ml}$  and total protein of 0.95mg.

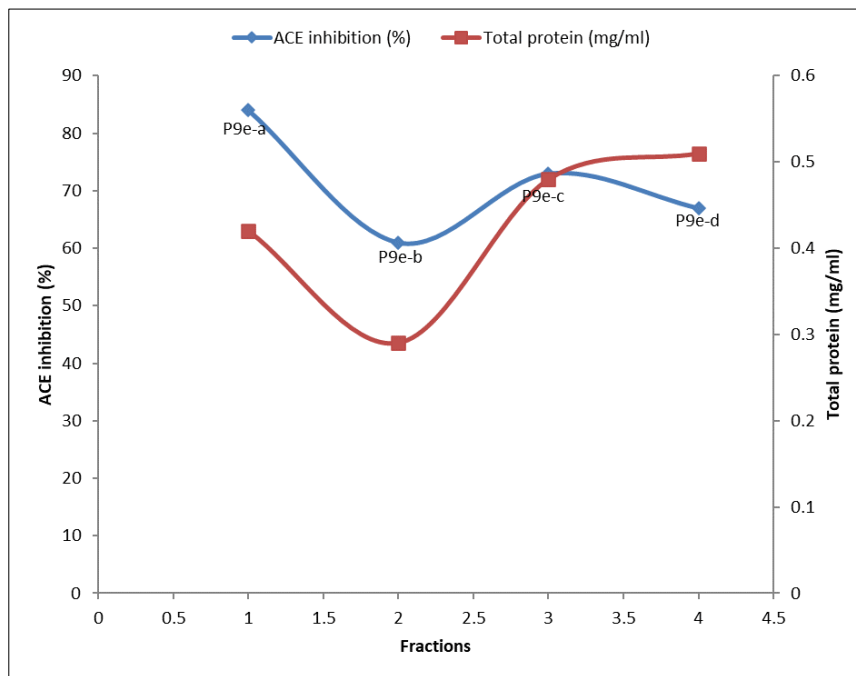


**Figure 2** Elution profile of partially purified ACE inhibitor from goat milk hydrolysate after the first fractionation by RP-HPLC.

From the third RP-HPLC purification step (Figure 4), four (4) active fractions were eluted (designated P9e-a to P9e-d) with fraction P9e-a having the highest ACE-inhibitory activity of 84% (Figure 4) with  $IC_{50}$  of  $3.25 \pm 0.11 \mu\text{g/ml}$  and total protein content of 0.42mg.



**Figure 3** Elution profile of partially purified ACE inhibitor from goat milk hydrolysate after the second fractionation by RP-HPLC.



**Figure 4** Elution profile of ACE inhibitor from goat milk hydrolysate after third step RP-HPLC.

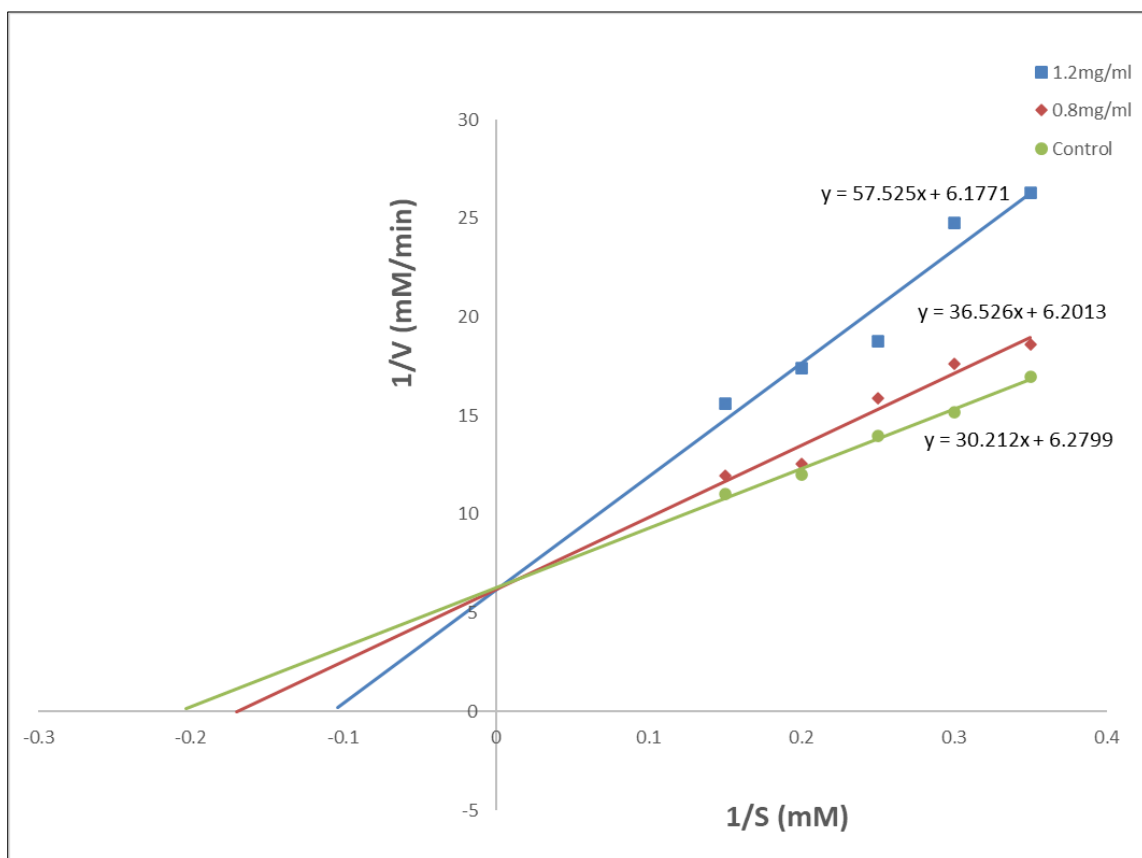
The pepsin hydrolysate was first treated to ultrafiltration through a 10-kDa molecular weight cut-off membrane, and this raised the ACE inhibitory activity from 57% to 62.7% with an  $IC_{50}$  of  $5.38 \pm 0.29 \mu\text{g/ml}$ . This is reliable with the findings of [22] and may imply that ultrafiltration removed high molecular weight peptides lacking ACE inhibitory activity [23] as low molecular weight peptides have been revealed to be more active antioxidants and antihypertensive peptides [24].

The findings of the present study showed that RP-HPLC enhanced the activity of ACE inhibitory peptide by eliminating components that may affect the activity of the peptide. There was a decrease in yield as the peptides were subjected to more purification (Table 1), the specific inhibitory activity and purification fold increased. This is in accordance with several studies (25,26,22).

### 3.3. ACE Inhibition Pattern of the Partially Purified Peptide

Using Lineweaver-Burk's plot, the ACE inhibition pattern of the partly purified peptide was assessed, and the peptide was shown to exhibit a competitive kind of inhibition, with no decrease or rise in  $V_{max}$  and an increase in  $K_m$  as inhibitor concentration rose. In the absence and presence of the isolated inhibitory peptide, the ACE activities were assessed. The partly purified peptide had the same  $V_{max}$  value (0.161) with no inhibitor, 0.8mg/ml inhibitor, and 1.2 mg/ml inhibitor. The  $K_m$  value increased from 4.8901 in the absence of the inhibitor to 5.8901 and 9.3126 in the presence of 0.8 mg/ml and 1.2 mg/ml of the inhibitor, respectively (Figure 5).

Enzyme kinetics is essential to understand the efficiency and inhibitory potential of peptides against enzyme activities. Lineweaver-burks' plot was used to analyze the pattern of inhibition of the partly purified peptide produced from goat milk hydrolysate. The plots (Figure 5) show that all of the lines crossed the y axis ( $1/V$ ) at the same point, showing that the  $V_{max}$  remained unchanged even in the presence of the inhibitor; instead, the concentration of the substrate required to reach half the maximum velocity ( $1/2V_{max}$ ) increased. While the value on the x axis decreased in the presence of the inhibitor, indicating an increased  $K_m$ , this result indicates that the inhibitory pattern of the peptide from goat milk hydrolysate is competitive with respect to HHL (Figure 5). This finding is consistent with other reports that most ACE inhibitors are competitive inhibitors, such as captopril [27] and *T. giganteum*, [28] fermented oyster sauce.



**Figure 5** Lineweaver-Burk's plot for the inhibition of ACE by the partially purified peptide purified from goat milk hydrolysate.

$1/V$  = initial velocity,  $1/[S]$  = Substrate Concentration,  $K_m$  = Michealis Constant,  $V_{max}$  = Maximum Velocity. Control = ACE Activity without Inhibitory Peptide.

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#### 4. Conclusion

The purified peptide isolated from goat milk hydrolysate possess potent ACE inhibitory activity, and may be used in the development of reasonably priced, efficient and safe antihypertensive peptides to serve as substitute to synthetic ACE inhibitory treatments but further studies is required to characterize the purified ACE inhibitory peptide.

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#### Compliance with ethical standards

##### *Disclosure of conflict of interest*

The writers have all stated that they have no conflicts of interest.

##### *Authors Contribution*

- Hashidu A.S. and Abdulazeez A.M. contributed to the study's conception, implementation, data analysis, and manuscript writing.
- Adekale I. A., Umar A. M. and Tyohemba T. S. contributed to data collection, processing and interpretation of the results. All of the writers examined the findings and approved the final manuscript version.

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