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Studies on Collagen Hydrolysis by Pineapple (*Ananas Comosus*) Stem Bromelain

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Abstract

The present study involves investigations on the collagen peptides obtained through bromelain hydrolysis. The collagen produced from Nitta Gelatin India Limited – Gelatin – H 1303080 was hydrolysed with bromelain extracted from pineapple stem. Collagen is the most important structural protein in the body, which gives the strength to build our body. Full length collagen molecules are broken down to form hydrolysed collagen. It is fat free, cholesterol free, easily digestible, and high in essential amino acids, hence used in medical field to help from sore joint recovery, cancer recovery, and post-surgery treatments. Initially, extracted bromelain was purified by ammonium sulfate precipitation, dialysis, and ion-exchange chromatography using DEAE cellulose column. SDS PAGE analysis showed the molecular weight of purified bromelain to be 30kDa. To separate bromelain from hydrolysed collagen, SEC method was employed. Homogeneity of these peptides was screened by using SDS PAGE. This led to the finding of 11 fractions of low molecular weight collagen peptides. The preliminary studies of hydrolysed collagen obtained through enzymatic hydrolysis were investigated. This study reveals that extracted stem bromelain was efficient for collagen hydrolysis.

Keywords

Collagen, Ion exchange chromatography, SDS-PAGE, SEC, Stem bromelain.

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INTRODUCTION

Collagen is the long-chain of amino acids that builds our skin, connective tissue and bones but cannot be absorbed through our diet. It is primarily made up of three amino acids- proline, hydroxyproline and glycine which give it unique functional properties different from all other proteins. It is the most important structural protein in the body, which gives the strength to build our body. Collagen is formed from three varied long chains of over 1,000 amino acids twisted helix

which gives the strength to build our body but is difficult to break down during digestion and too large to cross the intestinal wall. So, in its unhydrolyzed, full-length form, collagen is not an effective oral supplement [1]. Full length collagen is broken down into collagen peptides through a process called hydrolysis and are also frequently referred to as hydrolysed collagen [2]. Collagen peptides are more bio available and they better absorbed into the blood stream because they have much shorter chains of amino acids than collagen molecule. After absorption, they travel throughout the body, repairing, rebuilding, and providing energy [3]. Hydrolysed collagen used in skin-care products enhances the appearance of dry or damaged skin by reducing flaking and restoring suppleness [4]. Besides a few studies are reported on the collagen hydrolysis by using some proteases, no more reports are available on the collagen hydrolysis by using bromelain from pineapple stem. Bromelain is one of the protease enzymes found in pineapple plant, accumulated in the entire part with different extent and properties depending on its source. Stem bromelain (EC 3.4.22.32) is the major protease present in the extracts of pineapple stem while fruit bromelain (EC 3.4.22.33) is the major enzyme fraction found in the juice of the pineapple fruit [5]. It is included in the group of cysteine proteinases, have the ability to break peptide bonds, and hence to separate proteins and amino acids [6]. The continuing interest in bromelain, due to its numerous applications, both in food industry, and in the pharmaceutical industry, makes this enzyme one of the best proteases of vegetal origin. In the food industry, it is used in the clarification of beers, cheese making, softening of meat and in the preparation of hydrolysed collagen, in textile industries, for softening fibres and also for the production of detergents [7]. The present study deals with the enzymatic hydrolysis of collagen produced from Nitta Gelatin India Limited – Gelatin – H 1303080 with the extracted bromelain from pineapple stem.

MATERIALS AND METHODS

Chemicals and raw materials: Pineapple stem was obtained from a local area in Thavanur, Kuttippuram. Freeze-dried collagen was supplied by Nitta Gelatin India Limited – Gelatin – H 1303080, Kakkanad, Kerala. Macro-Prep® DEAE Media was purchased from Bio-Rad laboratories India PVT, Ltd. Sephadex™ LH – 20 gel was obtained from Amplicon BioLabs, Kakancheri, Chelambra. All the chemicals and reagents were of analytical grade.

Preparation of crude enzyme

100g of pineapple stem was blended with 0.1 M sodium phosphate buffer of p^H 7.0 containing 15 mM cysteine and 2 mM EDTA for 3minutes. Then filtered through a cheese cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes at 4^0c. The obtained supernatant was referred to as crude bromelain extract and was used for the experiments [8].

Purification of Extracted bromelain

Ammonium sulfate precipitation

Ammonium sulfate precipitation of the prepared extract was done according to the method described by S.S. Gautam and S.K. Mishra *et al.,* 2017 with some modifications [9].

Dialysis

Ammonium sulfate fraction of highest proteolytic activity was treated with dialysis to remove the effect of salt on bromelain purity. The solution was placed in a dialysis bag (pore size: 2.4nm, the treated tube retains the proteins of molecular weight greater than 14kDa). Then it was suspended in a beaker containing 100 mM phosphate buffer solution. This set up was kept in 4^0C at overnight with frequent changes of buffer [9].

Ion exchange chromatography of purified bromelain on DEAE-cellulose

After dialysis, stem bromelain was purified further with ion exchange chromatography using DEAE-cellulose as stationary phase. The dialyzed sample of stem was subjected to the Bio-Rad Biologic LP Protein purification system. Prior to injection, the column was equilibrated with 0.5 M sodium phosphate buffer of p^H 8.0. Enzyme was eluted radiantly with Tris - HCl buffer (p^H 8.0, 25 mM) containing NaCl with concentration rise gradually from 25 mM - 150 mM, and the flow rate was set at 0.5 ml/min [10].

Sodium dodecyl sufate – Polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of the purified bromelain was determined by using SDS-PAGE. This was done with 4% stacking gel and 15% separating polyacrylamide gel according to the method described by S.S Gautam *et al.,* 2010 with slight modifications [9].

Characterization of purified bromelain

Determination of protease activity

The protease activity of the bromelain extract was determined according to the method described in Murachi 1976 with slight modifications, using tyrosine as standard [11]. The bromelain activity was determined using casein (3% W/V) as substrate at p^H 7.5.

Determination of protein

Protein concentration of the sample was determined by using Bradford method and BSA was used as standard [12].

Specific activity of the enzyme

Specific activity of the enzyme was determined by dividing the enzyme activity in units with the protein concentration and expressed as U/mg.

Yield of purified enzyme

Yield of each purified fraction is the activity in percentage obtained, by dividing the total activity of the particular fraction with the total activity of crude enzyme.

Fold purification

Fold purification of each purified fraction was obtained by dividing the specific activity of that fraction with the specific activity of crude enzyme.

Collagen hydrolysis by using isolated bromelain

Low molecular weight bioactive peptides derived from the enzymatic hydrolysis of collagen were obtained from the procedure described by Zied Khiari & Rico D *et al., 2013* with some modifications [13]. One gram of freeze- dried collagen was mixed with 90 ml distilled water, and then homogenized for 1minute at room temperature. The solution was then heated to 80^0c for 10 minutes in a water bath to inactivate the endogenous enzymes. After cooling to 50^0c, the p^H of the mixture was adjusted to 8 using 1 N NaOH, and the volume was made up to 100 ml by adding water. Hydrolysis approach was done by adding isolated bromelain. For the enzymatic approach enzyme: substrate ratio was set to 1: 50 (W/W). Collagen was then hydrolysed for 24 hours under continuous shaking at 200 rpm. After hydrolysis, collagen hydrolysates were heated to 80^0c for 10 minutes to inactivate the enzymes, and then cooled at room temperature for 60 minutes. The sample was then centrifuged at 10,000 rpm for 20 minutes at 4^0c. The supernatant containing soluble collagen peptides was collected, and then stored at -20^0c.

Separation of low molecular weight collagen peptides from bromelain by using Size Exclusion Chromatography

The molecular weight distribution of the collagen peptides and the enzyme used for hydrolysis was estimated by using SEC method described by *Zied Khiari & Maurice* Rico D*., 2013* with some modifications. To observe the general molecular weight profile of the collagen peptides, 1 ml of collagen peptide sample was loaded on a sephadex™ LH – 20 packed column and eluted with 50 mM Sodium phosphate buffer containing 30 mM NaCl at p^H - 7.4. The detection of peptides was carried out at an absorbance of 214 nm [13].

Determination of the homogeneity of SEC fractions by using SDS – PAGE analysis

The homogeneity of the fractions obtained from size exclusion chromatography was determined through the evaluation of their protein pattern using SDS-PAGE analysis was performed by using the method by Zied Khiari & Rico D *et al., 2013* with some modifications [13]. SDS-PAGE was carried out using 4% stacking gel and 15% separating polyacrylamide gel for low molecular weight peptides.

RESULTS AND DISCUSSION

Crude extract and results of precipitation with ammonium sulfate

Bromelain was successfully extracted from pineapple stem. The stem bromelain from *Ananas comosus* was purified using 150 ml of crude extract. The specific activity of the crude extract was found to be 4.6875 ± 0.001 U/mg. Table 1 shows the highest activity in bromelain fraction can be found in 4^{th} fraction (60-80 %), which is 15.6090±0.062 U/mg with degree of purity 3.3229-fold in comparison with the crude extract, and yield of 88 %.

Table 1 Stem bromelain specific activity on evaluation steps of extraction and precipitation using Ammonium sulfate.

Samples	Specific Activity (U/mg)
Crude extract	4.6875 ± 0.001
*F 0-20%	2.4107 ± 0.034
F 20-40%	5.4307 ± 0.077
F 40-60%	7.3326 ± 0.076
F 60-80%	15.6090 ± 0.062

(*Fraction)

The values are averages of six independent experiments ± SEM.

In a previous study conducted on the stability test of a partially purified bromelain from *Ananas comosus* (L.) Merr core extract in artificial stomach fluid, the highest specific activity of bromelain fraction was in the range of 50-80 %, which is 14.591 U/mg [10]. So, as per the view of industrial application, the highest recovery yield was attractive for further studies. Therefore, the extracted bromelain obtained from 60-80% ammonium sufate saturation was chosen for investigating for the subsequent studies.

To achieve higher degree of purity, enzyme fraction with highest specific activity obtained during ammonium sulfate precipitation has been dialysed.

Result of Dialyzed fraction

After treating the ammonium sulfate fraction with dialysis, specific activity of the stem bromelain fraction has increased from 15.6090 ± 0.062 U/mg to 23.8429 ± 0.0009 U/mg, with degree of purity 5.0864-fold in comparison with the crude extract, and yield of 80 %. Table 2 shows the specific activity of stem bromelain before and after dialysis.

Table 2 Result of Dialyzed stem bromelain fraction

Samples	Specific Activity (U/mg)	Yield (%)	Degree of Purity
Before dialysis	15.6090 ± 0.062	88	3.3229
After dialysis	23.8429 ± 0.0009	80	5.0864

The values are averages of six independent experiments ± SEM.

Result of Ion Exchange Chromatography

After treating the dialyzed fraction with ion exchange chromatography, specific activity of the bromelain fraction has increased from 23.8429 ± 0.0009 U/mg to 36.70 ± 0.007 U/mg with degree of purity 7.8293-fold in comparison with crude extract. Figure 1 shows the elution profile of the dialyzed fraction by DEAE-cellulose column chromatography, the enzyme was eluted as a single peak in the fractions 01-03. The fractions were collected gradiently, and the active fraction with higher enzyme activity was pooled and subjected to SDS- PAGE analysis.

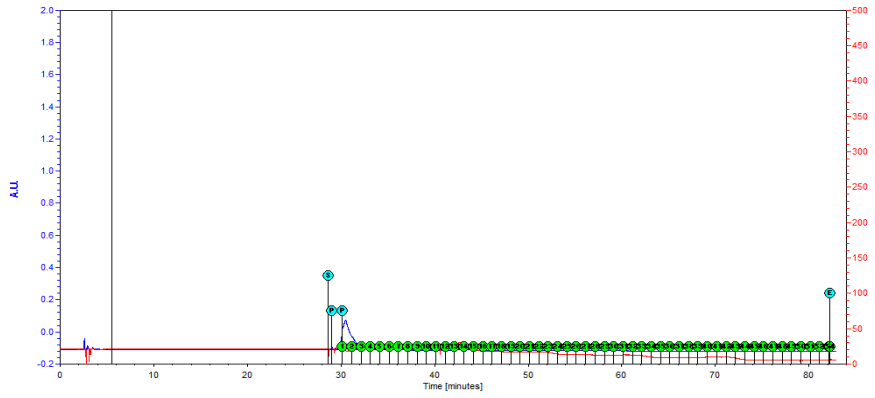

Figure 1 Chromatogram for 60 - 80 % Ammonium sulfate fraction using DEAE- Cellulose Matrix. The peak of protein was at 280 nm.

[Seperation conditions: Column size 5-15 mm ID, low-to-medium-presure lab columns, Matrix 20 % DEAE-cellulose, Flow rate was set at 0.5 ml/min. The elution process was done by using gradient method. The initial stage of the column was equilibrated with 0.5M sodium phosphate buffer of p^H 8.0. Subsequent elution was done with Tris-HCl buffer (p^H 8.0, 25 mM) containing NaCl with concentration rise gradually from 25 mM-150 mM].

Pineapple stem bromelain fractions obtained after each steps of purification gave an increasing factor of purity, as shown in Figure 3. The purification results are summarized in Table 3.

Table 3 Purification of stem bromelain from *Ananas comosus*

Sl. No	Steps of Purification	Specific Activity (U/mg)	Yield (%)	Degree of Purity
1.	Crude	4.6875 ± 0.001	100	1
2.	Ammonium Sulfate precipitation (60-80 %)	15.6090 ± 0.062	88	3.3229
3.	Dialysis	23.8429 ± 0.0009	80	5.0864
4.	Ion exchange chromatography using DEAE- cellulose	36.70 ± 0.007	53.33	7.8293

The values are averages of six independent experiments ± SEM.

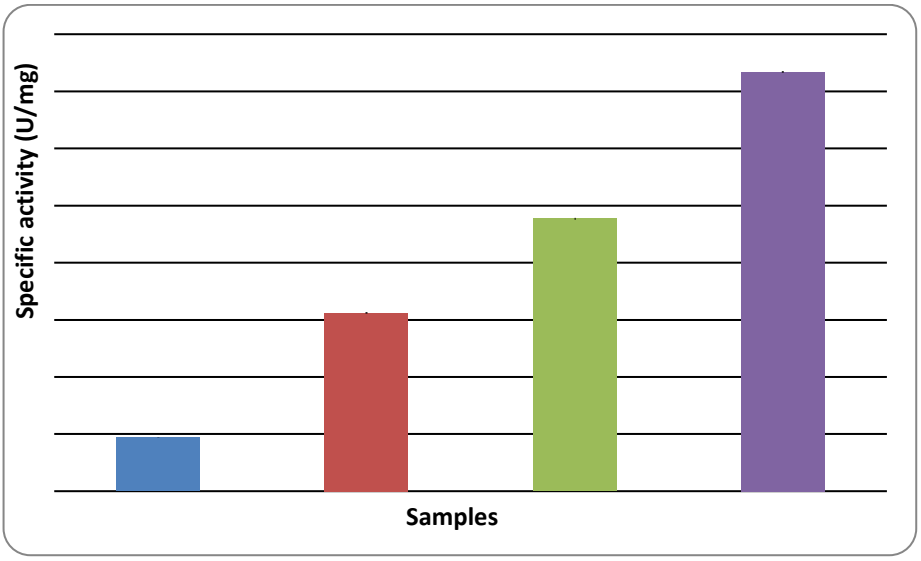

Figure 2 Specific activity of stem bromelain with each purification steps.

SDS – PAGE for molecular weight determination

The state of purity was determined at every successive stage of purification by using 15 % SDS-PAGE. The electrophoretic pattern of all the purified fractions obtained from ammonium sulfate precipitation, dialysis, and DEAE- cellulose ion exchange chromatography along with the crude extract is shown in Figure 3. From the SDS – PAGE analysis, an interesting finding was that the extracted protein (bromelain) was pure and stable during in-process of isolation steps (Figure 3). Moreover, single SDS-PAGE band of ion exchange isolated bromelain also inferred the integrity and purity of bromelain protein.

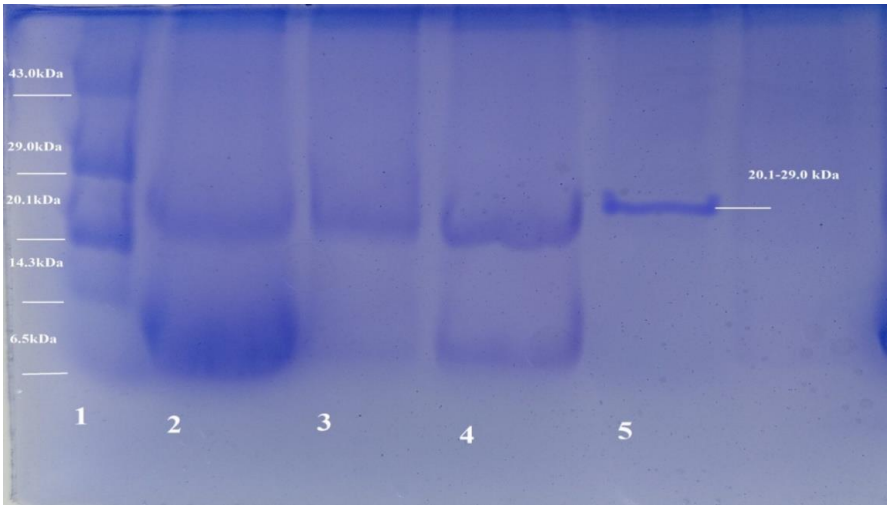

Figure 3: SDS-PAGE pattern of bromelain from pineapple stem.
Lane 1: Standard molecular weight, from top to bottom, Ovalbumin (43.0 kDa), Caebonic Anhydrase (29.0 kDa), Soyabean Trypsin Inhibitor (20.1 kDa), Lysozyme (14.3 kDa), Aprotinin (6.5 kDa).
Lane 2: Crude stem bromelain
Lane 3: Ammonium sulfate fraction (60-80 %)
Lane 4: Dialyzed sample

The estimated molecular weight of bromelain was around approximately 30 kDa as in previous report [6]. A study conducted by *Ketnawa et.al.* previously reported the main protein component found in bromelain extract of pineapple peel has molecular weight around 28kDa [8]. These previous reports are in agreement with our study. Protein pattern through SDS – PAGE showed the molecular weight of purified bromelain to be about 20-29 kDa.

Separation of low molecular weight collagenous polypeptides

The collagen produced from NITTA GELATIN INDIA LIMITED – Gelatin – H 1303080518 was hydrolyzed by using bromelain extracted from pineapple stem. Figure 4 shows the absorbance of the collagen peptides by using sephadex™ LH - 20 column at 214 nm.

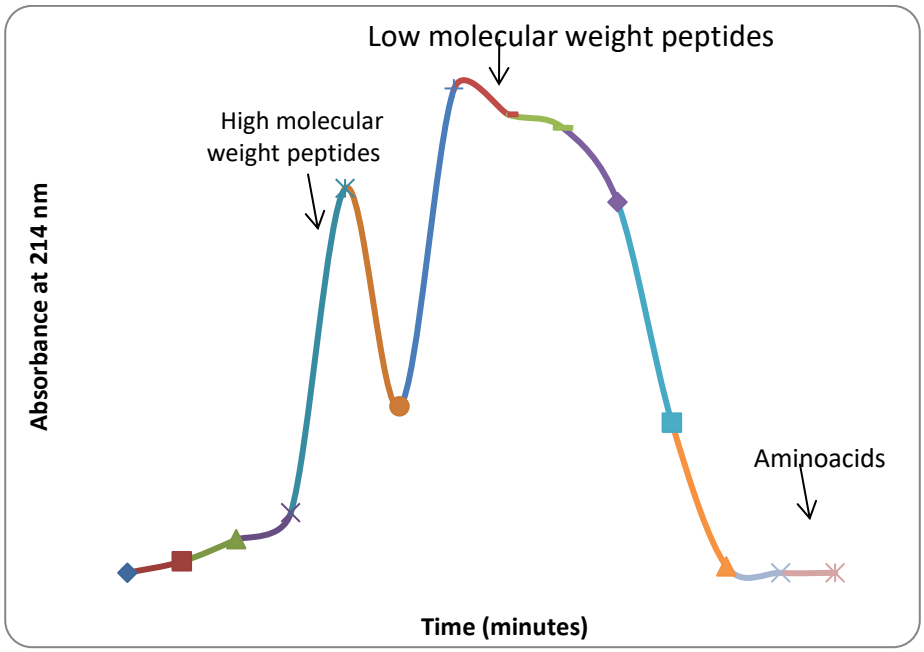

Figure 4: Elution profile of hydrolyzed collagen on treatment with extracted bromelain with Size Exclusion Chromatography. The peaks of peptides were at 214 nm. Peaks yielding peptide sequence are marked with arrows.

Determination of the homogeneity of SEC fractions

The result of homogeneity of the collagen peptides was determined through the evaluation of their protein patterns using SDS-PAGE analysis was shown in figures (5, 6). These figures (5, 6) showed that each fraction obtained from SEC exhibits electrophoretic protein bands, revealed that each fraction are peptide constituents. To observe the approximate molecular weight of SEC fractions, standard bromelain of molecular weight around 30 kDa was used.

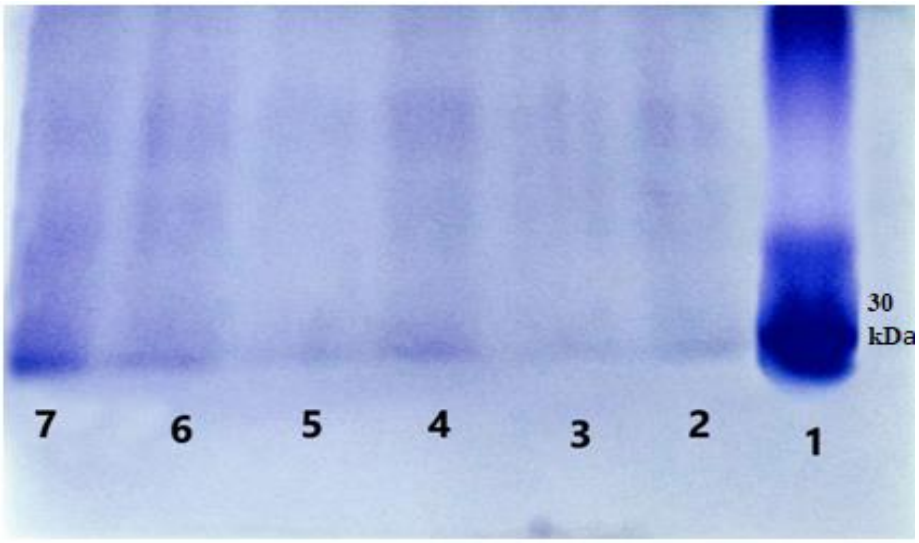

Figure 5: SDS-PAGE pattern of SEC fractions (1-6) by using 15 % resolving gel
Lane 1: Standard bromelain (molecular weight around 30 kDa)
Lane 2: Fraction 1, Lane 3: Fraction 2, Lane 4: Fraction 3, Lane 5: Fraction 4, Lane 6: Fraction 5, Lane 7: Fraction 6.

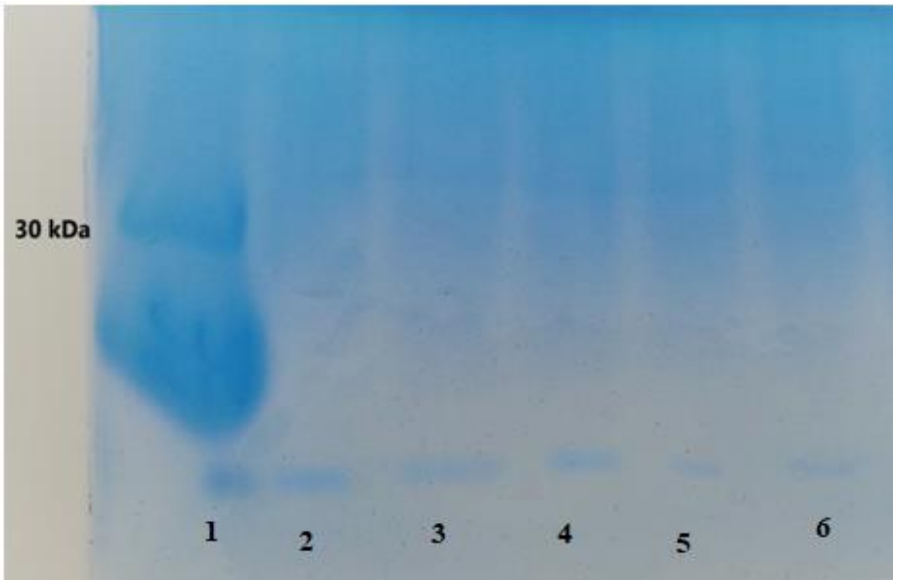

Figure 6: SDS-PAGE pattern of SEC fractions (7-11) by using 20 % resolving gel
Lane 1: Standard bromelain (molecular weight around 30 kDa)
Lane 2: Fraction 7, Lane 3: Fraction 8, Lane 4: Fraction 9, Lane 5: Fraction 10, Lane 6: Fraction 11.

The results inferred that, the molecular weight of each fraction was decreased on increasing number of fractions (Figure 5). It was found that, the extracted bromelain from pineapple stem has certain effect on collagen hydrolysis.

An identification study of food – derived collagen peptides in human blood after oral ingestion of gelatine hydrolysates, conducted by Iwai K & Hasegawa T *et al., 2005* reported that, the porcine type 1 gelatin hydrolysate consists of peptides with molecular weight from 1000 to 20,000 Da based on the elution volume from gel –filtration column [14]. According to the study conducted by Jeevithan E & Bao B *et al.* in 2015, proved that low molecular weight collagenous polypeptide (37 kDa) can be prepared from whale shark cartilage (*Rhincodon typus*) in digestion with thermolysin. Thermolysin specifically catalyzes the hydrolysis of peptide bonds containing hydrophobic amino acids [15].

By comparing our findings with the results of previous studies, we can see that the molecular weight of collagen peptides obtained through hydrolysis has some similarities. In our study, collagen is hydrolysed with bromelain extracted from pineapple stem, whereas in case of *Elango Jeevithan and Bin Bao et al.* they used thermolysin for hydrolysis approach.

Sunanta Ketnawa et al., [8] compared the peptide mapping of collagen from bovine Achilles tendon and giant fish catfish skin digested by stem bromelain. The bromelain extract was applied to hydrolyse the skin collagen of beef and giant catfish (0–0.3 units). The β, α_1, α_2 of giant catfish skin collagen extensively degraded into small peptides when treated with bromelain extract. Their study showed that bromelain from *Nang Lae* pineapple peel exhibited a high degree of collagen hydrolysis.

All of these identified collagen peptides were reported to have various biological activities and some of them were found to have antioxidant activity, anti-osteoporotic activity, anti-aging, anti-inflammatory, anti-tumour and wound healing activity [16]. More comprehensive study is needed to reveal the individual activities of these identified compounds.

Besides a few studies are reported on the collagen hydrolysis by using some proteases, no more reports are available on the collagen hydrolysis by using bromelain from pineapple stem. This enzymatic approach of collagen hydrolysis was almost agreeing with the earlier studies that are conducted with bromelain.

CONCLUSIONS

The preliminary studies of hydrolysed collagen obtained through enzymatic hydrolysis were investigated. The analysis led to the findings of 11 fractions of low molecular weight collagen peptides. This may be considered a further validation of hydrolyzed collagen. More comprehensive study is needed to reveal the individual activities of these identified compounds. The broad application of the present research is that use of hydrolyzed collagen in medical field. It could also investigate the association between different enzymatic approach like trypsin, pepsin etc on collagen hydrolysis. Looking forward, further attempts could prove quite beneficial to the literature. This provides a good starting point for discussion and further research.

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