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ISOLATION AND PURIFICATION OF B-CN FROM SHEEP MILK AND MEASURING THE EFFECTIVENESS OF ITS ENZYMATIC HYDROLYSIS IN INHIBITING ACE1

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Abstract

Milk is a biological important fluid that contains minerals, vitamins, fat, and proteins as crucial elements inside the human body that are used to support the body with natural supplements and might be used for therapeutic purposes. Purification of casein using salt and urea was used and it was partially purified using DEAE-Cellulose and then Sephadex-G-75. The resulting hydrolysate was estimated after incubation with pepsin, trypsin, and a mixture of them to study the inhibition rate of the Angiotensin-converting enzyme (ACE 1). β-CN was partially purified with one peak after DEAE-Cellulose ion exchange and there is also one peak that appeared after the Sephadex G-25 gel filtration technique. Maximum hydrolysis was gained after 8 hours of incubation using a mixture of pepsin and trypsin that inhibit ACE1 at an optimal level. The inhibition rate reached about 71% after 8 hours of incubation with a mixture of pepsin and trypsin with a hydrolysis concentration of 0.083μmol. Results showed that casein extracted from sheep milk can be highly purified using both ion exchange and gel filtration chromatography. Hydrolysis of α and β-CN produces low molecular weight protein using pepsin and trypsin and a mixture of them.

Keywords— β- Casein, Sheep milk, ion exchange, gel filtration, degree of hydrolysis, Angiotensin-converting enzyme

1. INTRODUCTION

Depending on the contents of each type of protein and its main structure, peptides can have multiple bioactive functions, including different hypotensive peptides (2). The protein casein is not homogenous. It is made up of many fractions, including s1-casein, s2-casein, beta-casein, and Kaba-casein (3). The ratios of the milk casein fractions vary depending on the species of ruminant, as well as their size, hydration, and mineralization. Casein from sheep's milk is more mineralized, less hydrated, and more heat stable (4).

Additionally, it is believed that milk proteins serve as the building blocks for a variety of biologically active peptides with better nutritional and immunological functions (5). Many disorders, including those caused by immune system deficiencies, thrombosis, mineral malabsorption, and hypertension, are treated with the use of bioactive peptides. Although there were differences in the casein components, mineral levels, and calcium to phosphate ratios in the sheep milk, all had remarkably high nutritional benefits for the human body (6).

The evidence that the peptide of peptide causes hypotension and the effectiveness of lowering blood pressure make the peptide research field one of the most important research fields in medical chemistry. This has led to serious concerns about the inhibition of ACE1 might be a drug as it directly affected on ACE1 inhibition (7). The kind and arrangement of the amino acids in peptide sequences determine the biological efficacy of low molecular weight compounds. Typically, bioactive peptides have between three to twenty amino acids per molecule (8).

Rare and restricted research has been done on the separation and hydrolysis of casein from local sheep milk to create peptides that have inhibitory effects on ACE1. (9), despite the significance of identifying the hydrolysis effect of their peptide on ACE1, revealed that there are very few articles on ACE-inhibition from hydrolysates gained from casein (CN) in sheep and goat milk. In order to create tiny molecular weight peptides that can reduce high blood pressure, this study purified the casein from sheep milk.

2. Materials and Methods

2.1 Reagents and enzymes

Fresh local sheep from Iraq that were obtained from the animal house directorate of the Ministry of Agriculture in Baghdad provided the Milk sample. used by operating the BL-S selecta centrifugal apparatus at 3000 g for 15 minutes and at 4 ci. It was done to separate the acidic casein from sheep milk. Both pepsin (Porcine Source, activity 2000u/gm, BDH business) and trypsin (Porcine Source, activity 1800u/gm, Merk company) are digestive enzymes. Bovine standard beta-CN was manufactured by Merk Company. BDH Company produced Sephadex G-75 Fine 45. Fluka Company's TNBS ACE1 (EC 3.4.15.1) (EC 3.4.15.1) Hippuric acid HA and Rabbit Lung.

2.2 -CN Separation

At the start of the separation process, 40gm of acidic casein that had previously been generated in 100 ml of urea was used.

To obtain 4.63M of the solution, 42.5ml of distilled water was added to the mixture, and preserved it at room temperature until white. The precipitate reduced the concentration of urea to 4.63M in 100 ml of a solution containing 0.326 molar NaCl and one precipitates which is beta-CN that has been stored in freeze drying.

2.3 β-Casein Purification with DEAE-Cellulose Ion Exchange Chromatography

According to (11) and (12), crude beta-CN that was previously separated from the precursors was purified using anion exchange chromatography (12).

beta- CN Phosphate buffer was used to dissolve the material, which contained NaCl in increasing concentrations of 0.1, 0.175, 0.2, and 0.25 molar. Protein solutions were pumped at a rate of 50 ml per hour at a concentration of 5 ml per tube, and the spectrophotometer, a Shimadzu model 1650 PC, read the concentration at 280 nm.

2.4 β- Casein Purification by Gel Filtration

As reported by the Pharmacia Fine Chemicals Company, the gel of Sephadex G-75 was used to construct columns. 0.5 grams of freeze-dried beta-CN that had been previously purified were dissolved in 10 milliliters of 0.005 phosphate buffer. The column of Sephadex with a dimension of (1.570×1) centimeter was eluted by a buffer containing the equivalent amounts of urea and EDTA. With a flow rate of 20ml per hour and 3 ml per tube, and protein solutions in tubes, fractions have been recovered from a Sephadex column and read at 280 nm by a spectrophotometer made by Shimadzu model 1650 PC. The volume of -casein was concentrated, and the Bradford method was used to measure the protein content (13).

2.5 Protein concentration assay

The standard beta-Casein was employed in the creation of the standard curve as shown in table 1, and the Bradford technique was used to estimate the concentration of beta-Casein in solutions. Estimate the molecular weight of beta-casein and confirm purity. The purity of beta-casein was determined using a vertical electrophoresis technique with polyacrylamide gel and sodium dodecyl sulphate (SDS) and its molecular weight was calculated. beta-CN with a known molecular weight using the method described in (14) and modified by (15). Protein-degrading enzymes break down casein. The adjustments to (9) were then applied in the ratio of the amount of protein and the quantity of proteolytic enzymw units to the quantity of protein acquired in the process of (16).

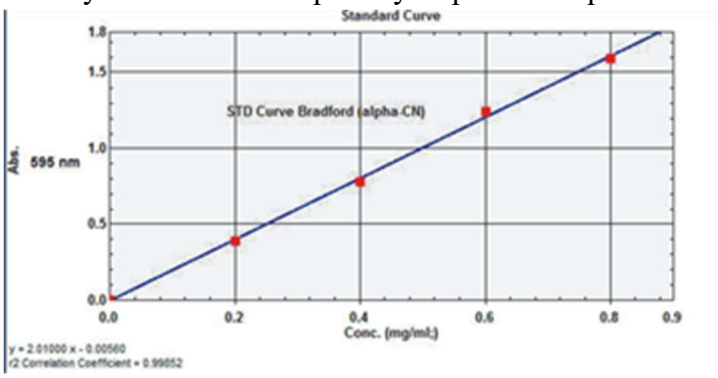

Figure1. Protein concentrations of are estimated β-CN using the Bradford method's standard curve at the absorption of 595 nm

Casein (0.1 g) was dissolved in 10ml of distilled water at pH 8 for trypsin, pH 2 for pepsin, and pH 2 then raised to pH 8 for the 1:1 mixture of the two enzymes. By adding Hcl and NaoH, the pH is altered. During the 8-hour reaction period at 37°C, pepsin and trypsin were added to the reaction mixture in increments of 20, and 18 units, respectively. Symbiotic enzymes reacted with pepsin and trypsin in a four-hour span, respectively. The degree of DH hydrolysis in beta-Casein is assessed using samples taken every hour.

2.6 Estimation of DH: The DH was calculated using (17). Leucine's standard curve, which is depicted in figure (2), was used to compute the DH of the samples. Each time, 0.2125 molar sodium phosphate buffer with a pH of 8.2 and 2 ml of 0.1% Tri nitrobenzene sulfuric acid (TNBS)

were added to 0.250 ml of the samples. The combination was then combined, and the mixture was incubated at 50 oC for an hour. The reaction was halted by adding 0.1 molar Hcl and let to cool to 30-2 oC for 30 min while being shielded from light. The absorbance was determined at 340 nm. The degree of hydrolysis (peptides concentration or Peripheral NH3 groups) was determined by plugging the values into the equation below. (18) using a Shimadzu instrument and the UV-PROBE 2.1 software.

$$DH = \left[\frac{L_t - L_0}{L_{MAX} - L_0}\right] \times 100$$

So that
Lt = peripheral NH3 group concentration at time (0-8 hr.).
L0 = Free amino acids (NH3 group) in original protein samples that have not been changed.
Lmax = L overall.
Lmax is the total amount of free amino acids produced after 24 hours of acidic hydrolysis by 6 molar Hcl at 120 cp.

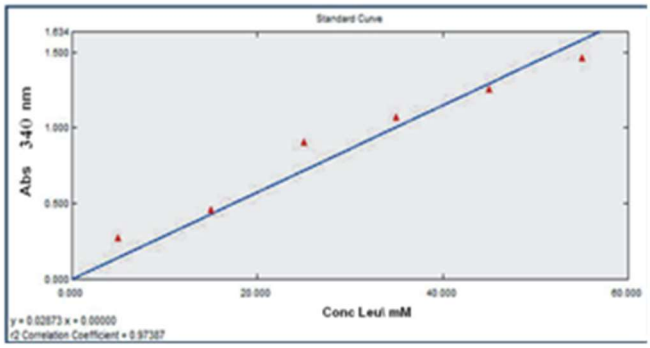

Figure 2. Leucine standard curve utilized for the degree of hydrolysis at 340 nm.

3. Results and Discussion

3.1 Acidic casein prepration and composition

During the season of 2021, milk samples from young sheep in the area were gathered from the field at the College of Agriculture/University of Baghdad. As a result, the solid non-fat content of sheep milk was 10.40%, and the moisture content was 83.2%. Additionally, it revealed that the level of protein in the local sheep milk was 5.3%, which was consistent with the study's findings that the total solid content of sheep milk was 16.8%. (15-20). Higher quantities of total solids (19.11%), fat (7.52%), protein (5.90%), lactose (4.55%), and solids-non-fat (11.45%) were also found in other trials (19). When compared to cow milk, sheep milk had a fairly close rise in fat content (6.4%). These numbers are regarded as extremely
Sheep milk had a density and refractive index of 1.045 g/cm3 and 1.354 g/cm3 respectively at 20 °C. These values exceeded the density and refractive index ranges (1.0347-1.0384 g/cm3) and (1.3492-1.3497 g/cm3), respectively, which may be attributable to variations in sheep breeds (19).

3.2 Extraction of crude Acidic Casein from sheep milk

Using cryogenic centrifugation and acidic precipitation, crude acidic casein was isolated from local sheep milk. The total amount of acidic casein protein in sheep milk was 4.5g/100g (43%), which was within the typical range of acidic casein protein in sheep milk (4.2-5.2g/100g), according to the results. The total amount of protein in milk sheep was 5.86g/100g (57%) and the total amount of protein in milk was. These findings are in line with recent studies (23) that revealed the minimal amounts of protein and casein in sheep milk for various breeds to be 5.5 and 4.7g/100g, respectively. The amount of protein in milk significantly affects its technological and nutritional value.

3.3 Purification of Primary β- Casein in sheep milk

The quantitative estimation of β-CN from total acidic sheep's milk casein yields 9g out of 40gm of total casein, which represents 22.5% of total Casein. These results agree with (24) who obtain 18% β-CN, On another hand, (23) showed that the amount of β-CN constitutes 61.6% of total casein.

3.4 Purification of β-CN in sheep milk using a DEAE-Cellulose anion exchanger

β-CN isolated from sheep milk was purified by Ion-Exchange chromatography using DEAE-Cellulose an anionic exchanger. Results are indicated in figure 3. Results illustrated in figure 3 showed that there is only one eluted peak in fraction numbers 15 to 35 using (0.4M) NaCl solution, and it belongs to β – CN because it has a less negative charge than αs– CN and K-CN protein, that was recovered from the column when using a (0.4M) NaCl solution due to the nature of its constituent acids, the two amino acids aspartic and glutamic, form the highest proportions of the rest of the acids in the β – CN molecule. Fractions representing β – CN were collected and pooled, then protein concentrations were estimated. The amount of the eluted β – CN was 125 ml that was used for the next purification step. The protein concentration was estimated (10.5g/ml).

Results indicated in Figures 3 showed that there is no interaction with another peak in the elution process which means that there is no other negative charge protein in the purified sample. These results disagree with (25) who showed that there is no peak in the wash step while there are four peaks that appeared after separation with the DEAE-Cellulose anion exchanger. Therefore, HPLC technique used to indicate the β-casein in the eluted sample.

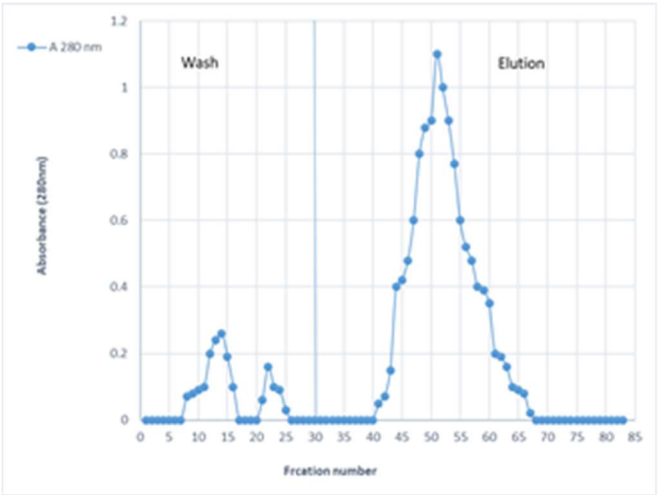

Figure 3. The purification of β - Casein sheep's milk on a DAEA-Cellulose column from 50x2 cm using a phosphate buffer of 0.5M molar, pH 7.4 containing a urea solution of 3.3 molars and 10 mmol of mercaptoethanol the binding fraction eluted using (a flow rate of 50 ml/hour, at a rate of 3 ml/tube, and with saline gradient NaCl from 0.1-0.9 M in the buffer).

3.4 Purification of β-CN by Gel Filtration Method by Using Sephadex G-75

Sephadex G-75 was the used to prepare the gel filtration chromatography column, according to the method used by (26) in purified β-CN milk with a modification in column length only, as gel filtration depends on the molecular weight differences between proteins (27). Results indicated in figure 4 showed that one peak was obtained in gel filtration chromatography. The peak appeared in tubes (11-35) when NaCl concentration was about 0.25M in phosphate buffer 0.05M. These results disagree with Kocher, (2018) who indicated that the elution process showed two major peaks. The first appeared in tubes (22-26) and the second in tubes 30-40. Fractions representing β-CN were pooled, then protein concentration was measured using Bradford method. agree with (28) and (29) who confirmed the possibility of obtaining one major protein peak for casein fractions of cow, sheep and goat milk by ion exchange method then gel filtration with a Sephadex column.

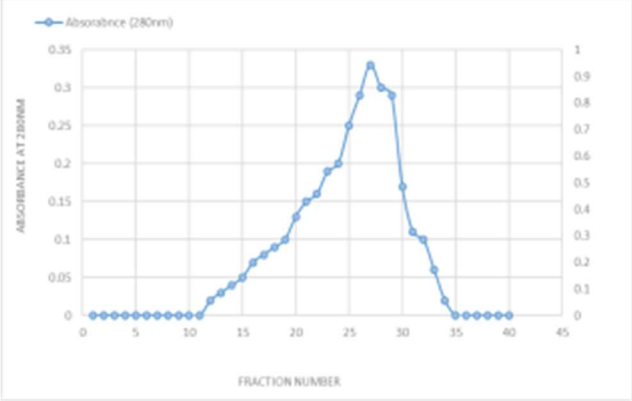

Figure 4. The purification of casein from sheep's milk using gel filtration chromatography required a sephadex G-75 column (90 x 1.5 cm) equilibrated with phosphate buffer saline pH 7.6 containing urea of 6.6 M and EDTA of 0.004 molar flow rate of 20 ml/hr, 3 ml/tube.

3.5 Purification of β- Casein using SDS-page

The purity and molecular weight of β-CN was determined using the SDS-PAGE method which depends on the electrophoresis of protein according to their charge. Results shown in figure 5 revealed that after electrophoresis of beta-CN, a single and purified beta-casein band emerged, insuring that it is devoid of additional casein fractions. The refined beta-CN also moved at similar distance as standard proteins, demonstrating the protein's purity. According to (30) and, alfa-CN and beta-CN is the prevalent in sheep milk casein" (31). In comparison to the mwt of the benchmark protein in Ladder, the mwt of pure -CN was 23 KD. The results agree with (32) showed that β-casein purified from sheep milk has a lower mwt (24.5) on SDS-PAGE. While (33) found that αs-CN was migrated to less than 26.8 KD in SDS-PAGE. β-caseins showed one crucial band

corresponding to a mwt of 14.4 kDa, and sheep αs-casein and β-caseins were degraded up to 76% and 19%, respectively, after 24 h of incubation (34).

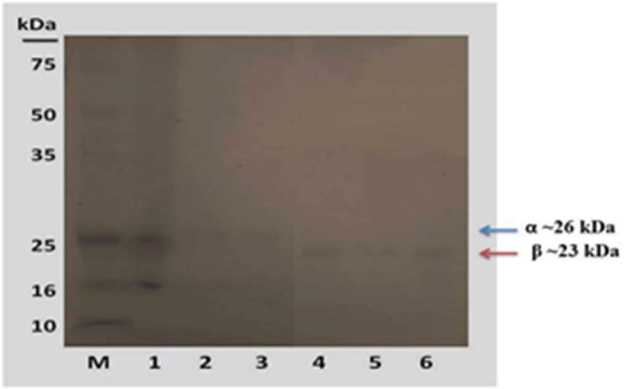

Figure 5. Migration of standard casein, standard ladder, and purified casein in 12.5% of SDS-PAGE. The standard casein in purified sheep milk is represented by β-23 KDa.

3.6 Hydrolysis of β- Casein

Hydrolysis of β-Casein in sheep milk was determined using Pepsin and Trypsin and a mix of both Enzymes for eight hours. Figure 6 of the data reveal that trypsin and pepsin treatment of β-CN increased the degree of hydrolysis (DH) due to high absorption values at 340 nm. In general, the three treatments behaved similarly, reaching maximum hydrolysis after 8 hours of incubation at 37°C, but in different proportions with Pepsin, trypsin, and their mixture, respectively. This indicates an increased release of peptides due to the specific hydrolysis effect of the enzymes. Results demonstrated that the optimum level of DH was obtained after incubation with a mixture of trypsin and pepsin after eight hours of incubation. These results were inconsistent with (25) who showed that DH enhanced to the highest level after incubation of pepsin and trypsin together to hydrolyze β-CN.

The results in Figure 6 indicated a high DH of β-Casein (61.0%), due to the efficiency of the synergistic action of the two enzymes which was similar to the DH of β-CN after incubation for 8 hours with trypsin and pepsin (1:1). These results agree with (35). Trypsin was used in the hydrolysis immediately after different proteolytic enzymes to increase the DH of casein from 44.5 to 72%. The high hydrolysis rates after purification of casein from sheep milk indicated that β-CN has low molecular weight peptides that can be obtained after hydrolysis according to the structure of β-CN, their biochemical components of amino acids and sequences, in addition to the type and concentration of enzymes used in the analysis, and the increase of β-casein decomposers by pepsin and trypsin in sheep (36). These results agree with (9) in goat milk casein, it means that there is a high hydrolysis efficiency of β-CN after incubation with pepsin, due to the availability of the basic cyclic amino acids and the specialized structure of sheep milk casein to stimulate the active sites in pepsin. On another hand, results indicated in figure 6 showed that the DH value of β-CN by trypsin:pepsin was 61.0% after 8 hours, which that maximum activity of hydrolysis was achieved

by the synergistic effect of pepsin: trypsin in hydrolysate of sheep milk, which agrees with (37) who reported that trypsin has less activity in β-CN.

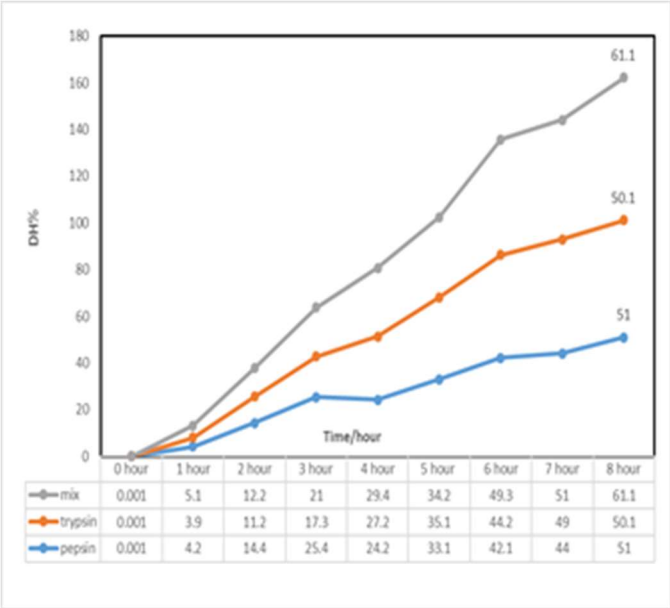

Figure 6. Hydrolysis% of beta-CN induced over an 8-hour incubation period at 37°C with a ratio of 1:1 mixture of pepsin and trypsin.

3.7 The Inhibitory Effectiveness of ACE1 by β–CN Hydrolysates (βH)

Results indicated in table 1 and figure 7 showed three hydrolysates after the determination of the inhibitory effect of β–CN Hydrolysates (βH) on ACE1. The β–CN Hydrolysates, which were hydrolyzed by the activity of pepsin, were incubated with ACE1 for three different time periods (1, 5, and 8 hours) which are referred to as β H1P, βH5P, and βH8P. In the same method, results also pointed out three hydrolysates by trypsinization of β– CN hydrolysates which are referred to as βH1P, βH5P, and β H8P in addition to three hydrolyses by the effect of a mixture of pepsin: trypsin after incubation for three different interval times (1, 5, and 8 hours) on ACE1 activity. ACE1 activity is determined by the measurement of HA concentration produced by the enzymatic activity of ACE1 on the hypuric acid substrate (HHL). Results indicated an inverse proportion among hip uric acid and the rate of inhibition% as shown in the figure 7. However, results demonstrated that there is an increased level of inhibitory rate in hydrolysate produced after 8 hours of incubation with pepsin, trypsin, and their mixture (66.263, 68.821, and 71.342 µMol respectively) than the other hydrolysate produced after 1 and 5 hours of incubation with the same hydrolyzing enzymes with the following concentration respectively (0.09, 0.075, and 0.084 respectively).

Results indicated in table 1 and figure 7 also showed that there is increased inhibitory effectiveness of ACE1 after 5 hours of incubation with pepsin, the inhibitory rate increased from 5.123% to 9.342% after incubation for 5 hours. While it increased from 8.532% to 17.632% after incubation with trypsin for 5 hours, the inhibition rate increased from 7.536% to 21.963% after incubation with a mixture of pepsin and trypsin for 5 hours. In this regard, the results showed that increasing the degree of hydrolysis is an important factor to raise the inhibitory activity due to the

emergence of new peptide chains that are characterized by low molecular weights and high inhibitory activity as a result of the continuation of hydrolysis processes.

On the other hand, due to the synergistic action of the pepsin and trypsin enzymes together, results indicated the importance of using a mixture of the two enzymes in the hydrolysis process to obtain the highest inhibition rate of ACE1. Results indicated in the table 1 showed that the highest inhibitory activity was 71.342% in βH8M with an HA concentration of 0.084 µMol that incubated for 8 hours with a mixture of pepsin and trypsin as indicated in table 1. This hydrolysate was collected for more purification processes to identify the specific peptide that is responsible for the inhibitory action.

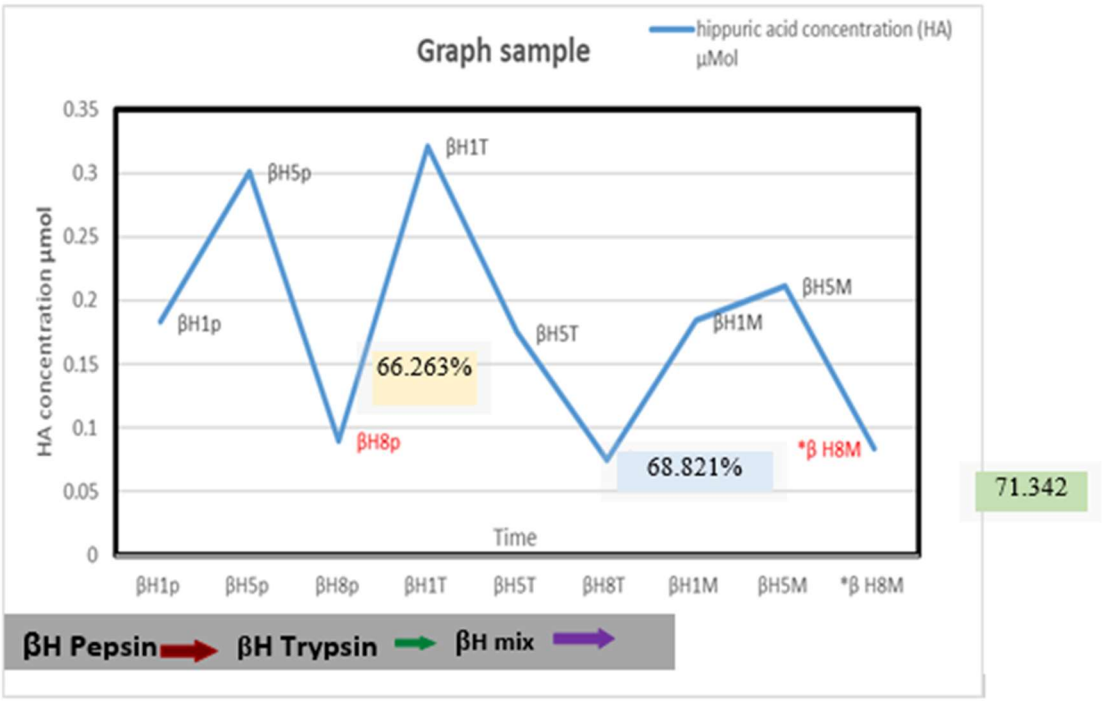

Figure 7. The role of βH of β-casein in ACE1 inhibition as formed by pepsin, trypsin, and their mixture at a 1:1 ratio.

These results agree with (38) which found the highest inhibition rate of ACE1 reached 67.6when analyzed for goat casein with trypsin while and (39) found that the inhibition rate of ACE1 by βH hydrolysate was 58% after 3 hours of incubation with trypsin. There are rare studies that demonstrate the inhibition rate of ACE in hydrolysates of sheep milk casein therefore, the study refers to (40) which showed that 12 peptides were identified, most of which originated from b-casein, that affected the inhibition of ACE1.

Table 1. The study of DH ratio and values of β-CN Hydrolysis concentrations with HA concentration and ACE1 inhibition efficacy.

β-CN hydrolysates	βH1p	βH5p	βH8p	βH1T	βH5T	βH8T	βH1M	βH5M	* βH8M
The inhibition activity%	z	9.342	66.263	8.532	17.632	68.821	7.536	21.963	71.342 *
hippuric acid concentration (HA) µMol	0.183	0.301	0.09	0.321	0.175	0.075	0.184	0.211	0.084
Hydrolysis degree%	5.5	31.32	51.424	7.2	32.543	45.235	6.432	31.345	59.235
Peptides Conc. (terminal NH3 group)	4.912	22.453	43.567	6.534	28.453	37.543	0.864	27.543	51.453

*: The hydrolysate had the highest ACE 1 inhibiting rate of β-CN

However, the βH hydrolysate form β-CN in sheep milk indicated that highest inhibition activity of ACE1 which was (71.342 %) with (0.084 µMol) of HA concentration which is high inhibition activity after incubation with pepsin and trypsin. These results disagree with (41) who proved that β- Casein hydrolyzers by trypsin have stronger activity than β-CN hydrolyzers with other enzymes in producing peptides effective in inhibiting ACE1, but (42) considered that there are two factors, hydrolysis degree and the type of peptides in casein hydrolyzer, affect on the emergence of high Inhibition activity in ACE1, which emphasizes the importance of synergistic action in releasing low molecular weight bioactive peptides. On another hand, It was demonstrated that sheep casein hydrolyzed by trypsin and pepsin had high ACE1 inhibition efficacy (67.1 and 61%, respectively), and that this activity increased with the degree of hydrolysis (DH), which was 48.0 and 59.5. It was also demonstrated that isolating peptides from the hydrolysates increased ACE1 inhibition activity to 97.6%. The most effective ACE1 inhibitory activity peptides that are also able to be taken orally are Val-Pro-Pro and Ile-Pro-Pro (9).

Conclusion

It concluded that β-CN can be highly purified using DEAE-Cellulose ion exchange and the Sephadex G-75 chromatography method. The molecular weight of β-CN is high. The synergistic action between trypsin and pepsin will obtain a high degree of hydrolysis of β-CN from sheep milk. The synergistic effect of pepsin:trypsin on βH was effective in the inhibition of ACE1, therefore this study might be a novel area to diagnosis the new bioactive peptide that is used for the treatment of blood pressure

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References

1. Mohapatra, A., Shinde, A. K., & Singh, R. (2019). Sheep milk: A pertinent functional food. Small ruminant research, 181, 6-11.
2. Ndiaye, F., T. Vuong, J. Duarte, R.E. Aluko and C. Matar (2012). Anti-oxidant, anti-inflammatory and immunomodulation properties of an enzymatic protein hydrolysate from yellow field pea seeds. European jour. of nutrition., 51(1): 29-37.
3. Bhat, Mohd Younus, Tanveer Ali Dar and Laishram Rajendrakumar Singh (2016). Casein Proteins: Structural and Functional Aspects. Chapter 1. INTECH. India.
4. Raynal-Ljutovac K, Lagriffoul G, Paccard P, Guillet I, Chilliard Y. 2008. Composition of goat and sheep milk products: An update. Small Rumin Res 79:57-72.
5. Punia H, Tokas J, Malik A, Sangwan S, Baloda S, Singh N, Singh S, Bhuker A, Singh P, Yashveer S, Agarwal S, Mor VS. Identification and Detection of Bioactive Peptides in Milk and Dairy Products: Remarks about Agro-Foods. Molecules. 2020 Jul 22;25(15):3328.
6. Dziuba B, Dziuba M. 2014. Milk proteins-derived bioactive peptides in dairy products: Molecular, biological and methodological aspects. Acta Sci Pol Technol Aliment 13:5-25.
7. Urgu M, Türk A, Ü nlütürk S, Kaymak-Ertekin F, Koca N. 2019. Milk fat substitution by microparticulated protein in reduced-fat cheese emulsion: The effects on stability, microstructure, rheological and sensory properties. Food Sci Anim Resour 39:23-34.
8. Meisel, H. and R.J. Fitz Gerald (2003). Bifunctional peptides from milk proteins: mineral binding and cytomodulatory effects. Current pharmaceutical design., 9(16): 1289- 1296.
9. Lee, K.J., S.B. Kim, J.S. Ryu, H.S. Shin and J.W. Lim (2005). Separation and purification of angiotensin converting enzyme inhibitory peptides derived from goat's milk casein hydrolysates. Asian-Australasian journal of animal sciences., 18(5): 741-746.
10. Zittle, C.A., J. Cerbulis, L. Pepper and E.S. Della Monica (1959). Preparation of calcium-sensitive □-casein. Journal of Dairy Science., 42(12): 1897-1902.
11. Whitney, R.M. (1988). Proteins of milk. In Fundamentals of dairy chemistry (81-169). Springer, Boston, MA.
12. White, A.J., S.C. Cheruvu, M. Sarris, S.S. Liyanage, E. Lumbers, J. Chui, D. Wakefield and P.J. McCluskey (2015). Expression of classical components of the renin-angiotensin system in the human eye. Journal of the Renin-Angiotensin- Aldosterone System., 16(1): 59-66.
13. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72(1-2), 248-254.
14. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680- 685.
15. Schägger, H. (2006). Tricine-SDS-PAGE. Nature protocols., 1(1): 16.
16. Adamson, N.J. and E.C. Reynolds (1996). Characterization of casein phosphopeptides prepared using alcalase: Determination of enzyme specificity. Enzyme and microbial technology., 19(3): 202-207.

17. Liu, B.L. and P.S. Chiang (2008). Production of hydrolysate with antioxidative activity and functional properties by enzymatic hydrolysis of defatted sesame (Sesamum indicum L.). International Journal of Applied Science and Engineering., 6(2): 73-83.
18. Jamdar, S. N., Rajalakshmi, V., Pednekar, M. D., Juan, F., Yardi, V., & Sharma, A. (2010). Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. Food Chemistry, 121(1), 178-184.
19. Vesna, P., Neven, A., Mioč, B., Ivanković, A., and Havranek, J. (2002). Influence of stage of lactation on the chemical composition and physical properties of sheep milk. Czech Journal of Animal Science, 47.
20. Paszczyk B, Łuczyńska J. The Comparison of Fatty Acid Composition and Lipid Quality Indices in Hard Cow, Sheep, and Goat Cheeses. Foods. 2020; 9(11):1667.
21. Kocher J. Ibrahim and Jasim M. Salah Al-Saadi, (2018). Effect of storage time on physiochemical and sensory properties of Karadi sheep milk yoghurt. JZS, Special I
22. Matutinović, S., Rako, A., Tudor Kalit, M., and Kalit, S. (2021). Influence of the Ratio of Sheep and Cow Milk on the Composition and Yield Efficiency of Lećevački Cheese. Fermentation, 7(4), 274.
23. Balthazar, C., Pimentel, T., Ferrão, L., Almada, C., Santillo, A., Albenzio, M., Silva, M. (2017). Sheep milk: physicochemical characteristics and relevance for functional food development. Comprehensive reviews in food science and food safety, 16(2), 247-262.
24. Garau, V., Manis, C., Scano, P., & Caboni, P. (2021). Compositional characteristics of mediterranean buffalo milk and whey. Dairy, 2(3), 469-488.
25. AL-Shammary O.A. and Dosh K.S., (2020). isolation and purification of □s2-cn local goat milk and studying its hydrolysates inhibition efficacy towards ace1. Plant Archives. Volume 20 No. 1, 2020 pp. 1917-1925.
26. Al-Saadi, J. M., and Deeth, H. C. (2008). Cross-linking of proteins and other changes in UHT milk during storage at different temperatures. Australian Journal of Dairy Technology, 63(3), 93.
27. Sandra Vairo Cavalli, Sofia V. Silva, Cecilia Cimino, F. Xavier Malcata, Nora Priolo, (2008). Hydrolysis of caprine and ovine milk proteins, brought about by aspartic peptidases from Silybum marianum flowers, Food Chemistry, Volume 106, Issue 3, Pp: 997-1003,
28. Ruprichova, L., G. Tompa, M. Kralova, I. Borkovcova, I. Bedanova and L. Vorlova (2015). Profiling of caseins in cow's, goat's and ewe's milk and dairy products by reversed-phase high-performance liquid chromatography. Journal of Food and Nutrition Research., 54(3).
29. Vincenzetti, S., P. Polidori, E. Salimei, P.L. Mariani, S. Allegri, N. Cammertoni, B. Quadrini and A. Vita (2005). Purification and Identification of □S1and □-Caseins from Asses Milk. Veterinary research communications., 29(2): 211-213.
30. Anema, S.G. and D.J. Stanley (1998). Heat-induced, pH- dependent behaviour of protein in caprine milk. International Dairy Journal., 8(10-11): 917-923.

31. Moioli, B., F. Pilla and C. Tripaldi (1998). Detection of milk protein genetic polymorphisms in order to improve dairy traits in sheep and goats: a review. Small Ruminant Research., 27(3): 185-195.
32. Khalil I. Ereifej, Muhammad H. Alu'datt , Hana A. AlKhalidy, Inteaz Alli, Taha Rababaha, (2011). Comparison and characterisation of fat and protein composition for camel milk from eight Jordanian locations. Food Chemistry 127 (2011) 282–289.
33. Raak, N., Abbate, R., Lederer, A., Rohm, H., & Jaros, D. (2018). Size separation techniques for the characterisation of cross-linked casein: A review of methods and their applications. Separations, 5(1), 14.
34. Tsabouri, S., Douros, K., & N Priftis, K. (2014). Cow's milk allergenicity. Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders), 14(1), 16-26.
35. Sun, H., T.J. Li and X.H. Zhao (2014). Ace Inhibition and Enzymatic Resistance In Vitro of a Casein Hydrolysate Subjected to Plastein Reaction in the Presence of Extrinsic Proline and Ethanol-or Methanol-Water Fractionation. International journal of food properties., 17(2): 386-398.
36. Jasińska, B. (1995). The comparison of pepsin and trypsin action on goat, cow, mare and human caseins. Roczniki Akademii Medycznej w Bialymstoku (1995), 40(3), 486-493.
37. Rodriguez, J., Gupta, N., Smith, R. D., & Pevzner, P. A. (2007). Does trypsin cut before proline?. Journal of proteome research, 7(01), 300-305.
38. Lei Wang, Nanxi Wang, Wenping Zhang, Xurui Cheng, Zhibin Yan, Gang Shao, Xi Wang, Rui Wang, Caiyun Fu. (2022) Therapeutic peptides: current applications and future directions. Signal Transduction and Targeted Therapy 7:1.
39. Otte, J., Shalaby, S. M., Zakora, M., & Nielsen, M. S. (2007). Fractionation and identification of ACE-inhibitory peptides from α-lactalbumin and β-casein produced by thermolysin-catalysed hydrolysis. International Dairy Journal, 17(12), 1460-1472.
40. Tiantian Tian, Wansheng Xie, Luxuan Liu, Siting Fan, Heqian Zhang, Zhiwei Qin, Chao Yang. (2021) Industrial application of antimicrobial peptides based on their biological activity and structure-activity relationship. Critical Reviews in Food Science and Nutrition 0:0, pages 1-16.
41. Ferreira, I. M. P. L. V. O., Pinho, O., Mota, M. V., Tavares, P., Pereira, A., Goncalves, M. P., and Teixeira, J. A. (2007). Preparation of ingredients containing an ACE-inhibitory peptide by tryptic hydrolysis of whey protein concentrates. International Dairy Journal, 17(5), 481-487.
42. Munn, S. M. (2013). Dairy and Soy Derived Bioactive otensin Peptide and the Renin-Angiotensin-Aldosterone System. A thesis submitted to the faculity of the graguate school of the university of Minnesota for the degree of master of science.