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ISOLATION AND PURIFICATION OF α_{S2}-CN LOCAL GOAT MILK AND STUDYING ITS HYDROLYSATES INHIBITION EFFICACY TOWARDS ACE1.

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Abstract

Hypertension is a universally common chronic disease and is called Silent Killer Current trend is to discover natural alternatives instead of chemical medications to treat this disease. In this study hydrolysates from goat milk casein have been used as natural materials to inhibit activity of Angiotensin Converting Enzyme1 (ACE1) the enzyme which is responsible on hypertension. α_{S2}-Casein from acidic local goat milk casein has been purified utilizing anion exchanger DEAE-Cellulose then by gel chromatography using sephadex G-75, electrophoresis technique SDS-PAGE Proven existence one pure type of α_S-CN is α_{S2}-CN which has been appeared as one band and has mw 25.620 KD.

Purified α_{S2}-CN has been hydrolyzed by pepsin, trypsin and mix them by 1:1. The hydrolysates appeared dissimilar ACE1 inhibition activity but highest rate 64.019 % and highest degree of hydrolysis (DH) 61.924 with hydrolysate produced by mix of tow enzymes after 8 hours of hydrolysis αH8M compared with inhibition activity by pepsin αH8P and trypsin αH8T after 8 hour too which were 54.146, 50.033 % and DH 48.293, 37.753 respectively. Inhibition activity towards ACE1 is directly proportional with DH value and hydrolysis period.

Key words: α_{S2}-CN, Goat milk, ACE1

Introduction

According to last statistics of World Health Organization WHO about 17.1 million person around the world are infected with Hypertension yearly. There are many medications and chemical therapies treat this disease most of them focus on inhibit producing the responsible article of hypertension referred to Angiotensin2 (Ang2) which converted from Ang1 by activity of ACE1 in humans and experimental animals (Sica, 2005).

Chemical angiotensin converting enzyme1 inhibitors (ACE_1I_s) have side effects in some body organs like kidney, lungs and liver like Symptoms of dry cough caused by tightness of the Pulmonary vesicles and angioedema (Guyer and Banerji, 2015). At recent days the world turned to natural materials to treat many diseases including high blood pressure. The world has turned to the use of natural materials to treat a number of diseases, including high blood pressure, most of which focus on inhibiting the effectiveness of ACE1 within Renin-Angiotensin-system (RAS) (AL-khafaji, 2008; Laragh, 2001).

ACE (EC3.4.15:1) is a dicarboxypeptidase type and metalloenzyme it's active side containing zinc ion and there is tow forms of this enzyme ACE1 and ACE2, the difference between them in stimulation sites. The main ACE1 function is cutting dipeptide (HIS-LEU) from carboxylic side of peptidyl hormone Ang1 consisting of 10 amino acids to convert it to Ang2 which is consider vasoconstrictor hormone, it presents in all blood vessels and it affects in secretion aldosterone hormone in kidney which causes hypertension so that referred to this regulatory role system "Renin- Angiotensin- Aldosterone System" (RAAS), ACE1 found in several human body organs but its highest concentration in pulmonary tissue (Aluko, 2018).

In several studies it was noted that in 1960 the English scientist Sir John Vane discovered a peptide from the Brazilian arrowhead viper, this peptide has the ability to rapidly lower blood pressure. It was later concluded that this peptide inhibits the ACE and this inhibition is fiercely competitive, this led to make an inhibitors similar to but non-peptide synthetic inhibitor and the first medication was CAPTOPRIL which contains sulfhydryl group and

many other medications but all of them have side effects (Brown and Hall, 2005; Wong *et al.*, 2004).

The peptide research field is one of the most important research fields of preventive medical chemistry since there have been medical evidence that the lack of peptide leads to hypotension and the effectiveness of reduced blood pressure, this led to the important question that the effect comes from inhibition of ACE1 only as chemical drugs inhibit or inhibition enzymes in and the answer was "Mostly with a direct and obvious effect in ACE1" Globally, there are many products containing hypotensive peptides in the commercial markets and are also included in the pharmaceutical formulations, mainly sesame peptides, seaweed peptides, peanuts, and sour milk milk. Most of these products, if not all, inhibit the effectiveness of ACE, other useful functions of the human and animal organs, most notably antioxidant and antimicrobial peptides, these properties are often found in the bioactive peptides (Shi *et al.*, 2014; Aluko, 2012).

Biologically and physically, the bioactive peptides are a sequence of amino acids which are affiliated within the protein and has positive functional and therapeutic properties within the human body but these properties appears only when it is released from the original protein by several hydrolysis ways like lipolysis enzymes of digestive tract and food fermentation by bacteria especially lactic acid bacteria (LAB) or exposing proteins or their isolates are to single or mixed proteolysis enzymes extracted from microbial, animal or plant sources (Munn, 2013; Korhonen and Pihlanto, 2007). Milk Protein one of the proteins which can be released with distinct hypotensive peptides and that peptides can have more than one bioactive function type of these peptides depend on the composition of each type of proteins and its primary structure, which determines the extent of resistance to the proteolysis enzymes of protein, especially enzymes that work in digestive tract. (Ndiaye *et al.*, 2012). The biological effectiveness of low molecular weight molecules is based on the type and sequence of the amino acids forming the peptide sequence. Bioactive peptides usually contain a number of acids ranging from 3 to 20 amino acids per molecule (Meisel and Fitz, 2003).

Studies on the effect of peptides and fermentation factors from goats' milk that have inhibitory effect on ACE1 are rare and are limited the most are focus on fermentation processes using different strains and Evaluation their inhibition efficacy on ACE1 and reducing blood pressure. In an experiment on this subject, 28 strains of lactic acid bacteria were used to ferment goat's milk, the *in vitro* results of the experiment showed that there were 20 types of ACE1 efficacy inhibitors, but only four showed very high efficacy in ACE1 inhibition rates by probiotic *Lactobacillus* .spp were 95.92, 84.61, 82.79, and 78.57%. Another study the ACE1 inhibition activity was 91.6% by goat milk fermentation with *lactobacillus planetarium* 691 (Chen *et al.*, 2018; Chen *et al.*, 2012).

Lee *et al.*, (2005) mentioned that a Little studies can be found on ACE-inhibition from proteolytic hydrolysates derived from casein (CN) in goat's milk, his experiment showed that goat casein hydrolyzed by pepsin and trypsin show up high ACE1 inhibition efficacy which were 67.1, 61% respectively and this activity increases with the degree of hydrolysis (DH) which were 48.0 and 59.5 ¡conclusion that isolation peptides from its hydrolysates cause increasing in ACE1 inhibition activity to 97.6%. Val-Pro-Pro and Ile-Pro-Pro are the most successful ACE1 inhibition activity peptides resistant to the digestive enzymes so that can be consumed orally. They are originally derived from the hydrolysis of β-CN in cow's milk and proved to be found in various types of solid, semi-solid and soft cheeses (Rutella *et al.*, 2016).

Many other researches proved ACE1 inhibition activity of hydrolyzed caseins and whey protein by digestive enzymes in addition to systolic blood pressure reduction in spontaneously hypertensive rats (SHR) comparing with treatment by chemical drug (captopril) (Sun *et al.*, 2014; Costa *et al.*, 2005). Many researchers, including (Aslam *et al.*, 2018; Bansal and Bhandari, 2016), suggest that the active peptides resulting from the hydrolysis of pure α, β and κ-CN and have molecular weights lower than the molecular weight of the peptides resulting from the hydrolysis of complete casein molecules when used Pepsin, trypsin and chymotrypsin with a difference of up to 85% in β-CN and 75% in α_S-CN. This is important in the production of bioactive peptides, especially antioxidants and inhibitors of ACE1. RP- HPLC is very effective in separating peptides containing hydrophilic amino acids. The results were also observed with the hydrolysis of the whey protein compared to hydrolysis of its previously separated fractions by chromatographic methods and by the use of the same enzymes.

Materials and Methods

Enzymes and reagents

Fresh local Iraqi goat milk purchased from animal wealth directorate-ministry of Agriculture\Baghdad-Iraq defatted by using the BL-S selecta centrifugal device at 3000×g for 15 minutes and at 4°C. The separation of goat's milk acidic casein was performed according to the methods of AL-saadi, (2001). Trypsin (Porcine Source, activity 1800 u/gm, Merk company) and pepsin

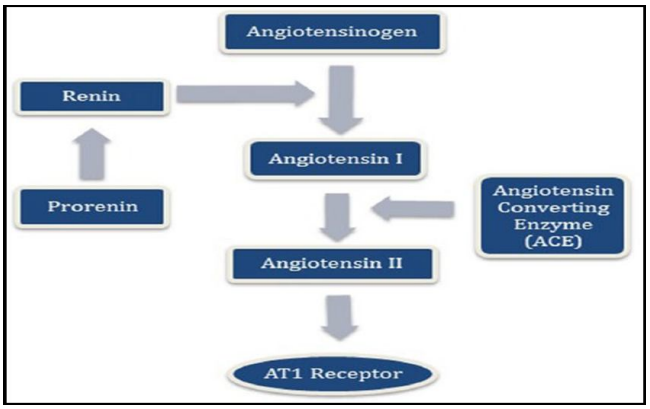


Fig. 1: Schema illustrates the role of ACE1 in Ang2 formation within RAS (White *et al.*, 2015).

(Porcine Source, activity 2000u/gm, BDH company). Standard bovine α_S-CN was from Merk Company. Sephadex G-75 Fine45 was from BDH Company. TNBS from Fluka Company. ACE1 (EC 3.4.15.1) From Rabbit Lung and Hippuric acid HA.

α_S-CN Separation

Method of Zittle *et al.*, (1959) followed up for α_S-CN separation from acidic casein with modification in acidic casein quantity which used in the beginning of the separation procedure 40gm of acidic casein previously prepared in 100 ml of urea 6.6 molar solution with stirring. Add 42.5 ml of distilled water to the solution to obtain 4.63 molar of urea solution and leave it at room temperature until forming white Precipitate which dissolved in 100ml of urea solution containing 0.326molar NaCl and reduction urea concentration to 4.63molar, the precipitate composed is α_S-CN which has been kept in freeze drying.

α_S- Casein Purification by Ion Exchange Chromatography

Anion exchange chromatography has been used to purify crude α_S-CN which separated from caseins previously according to Whitney, (1988). 1gm of freeze dried α_S-CN has been dissolve in 100ml sodium phosphate buffer 0.1molar and pH 7.4 containing urea 3.3 molar and mercaptoethanol 0.010 molar then mix with DEAE-Cellulose for 15 min. Aon exchange mix poured in column with dimensions of 2×50cm. The column washed by buffer solution conjugated caseins with column and α_S-CN Removed from column by using phosphate buffer which was used to dissolve sample containing NaCl in rising concentrations 0.1, 0.175, 0.2, 0.25 molar. Flow rate was 50ml/hr., 5ml/tube using fraction collector proteins solutions in tubes was read by spectrophotometer Shimadzu model 1650 PC on 280nm.

α_S- Casein Purification by Gel Filtration

Sephadex G-75 gel has been used to prepare column according to Pharmacia fine chemicals company. 0.5gm of freeze dried α_S-CN which purified initially by previous step dissolved in 10ml 0.005 molar phosphate buffer. Sephadex column with dimension 1.570×1 cm balanced by the same buffer containing urea 6.6 molar and EDTA 0.004 molar. Fractions has been recovered from Sephadex column with flow rate 20ml /hr. and 3ml /tube and proteins solutions in tubes read by spectrophotometer Shimadzu model 1650 PC on 280 nm. Total volume of α_S-CN was concentrated and protein concentration estimated by Bradford method.

Protein concentration assay

Bradford method was used to estimate α_S-CN concentration in solutions by using standard α_S-CN in standard curve preparation.

Confirm Purity and Estimate the Molecular Weight of α_S-CN.

To confirm the purity of αs-casein and determine its molecular weight, Method of electrophoresis with polyacrylamide gel was used in the presence of Sodium Dodecyl Sulphate (SDS) by using a vertical electrophoresis device comparing with standard bovine Known molecular weight α_S-CN based on the method described by Laemmli, (1970) and modified by Schägger (2006).

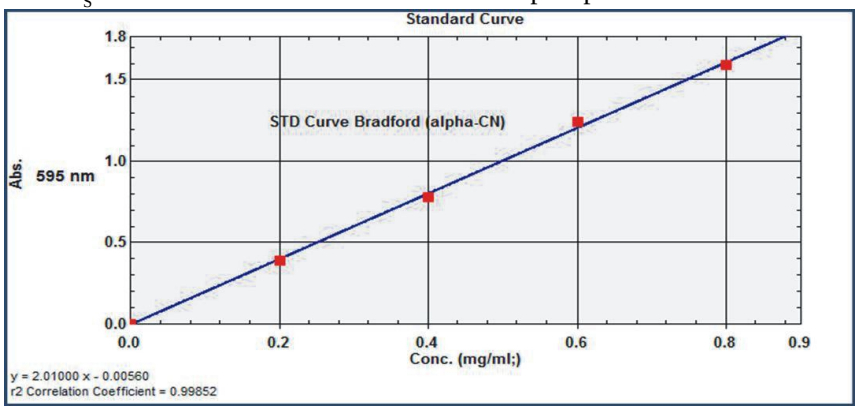


Fig. 2: αs-CN Standard curve of Bradford method used to estimate protein concentrations. The absorbance on 595 nm.

Hydrolysis of α_S-Casein by Proteolytic enzymes.

The method of Adamson and Reynolds, (1996) was followed with the modifications of Lee *et al.*, (2005) in the amount of the protein and the number of enzymatic proteolytic units in proportion to the amount of protein obtained from the purification steps. Weight of 0.1gm α_S-CN dissolved in 10 ml distilled water with pH 2 for pepsin

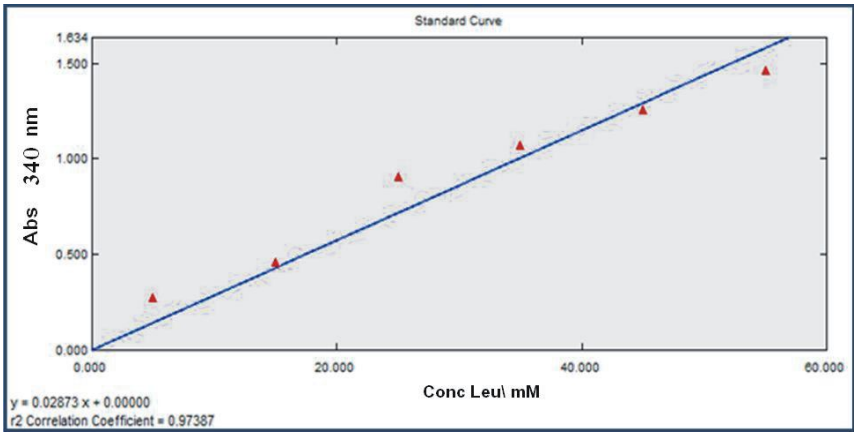


Fig. 3: Standard curve of Leucine used for DH%. The absorbance at 340 nm.

and pH 8 for trypsin and pH 2 then raising to pH 8 for mix of pepsin and trypsin in the rate of 1: 1. Changing pH by adding Hcl and NaoH. Pepsin and trypsin was added to reaction mix as 20, 18 unit respectively, reaction time was 8hr., at 37°C . Reaction time of symbiotic enzymes was 4hr., to pepsin and then 4hr., to trypsin. Samples pulled each hour to determine degree of hydrolysis DH.

Estimation of Degree hydrolysis (DH)

Degree of hydrolysis was estimated according to Liu and Chian (2008). Standard curve of amino acid leucine (Fig. 2) was used to calculate DH of samples. Volume of 0.250ml of samples for each time was transferred to test tube and mix with sodium phosphate buffer 0.2125 molar with pH=8.2 and 2ml of 0.1% Tri nitrobenzene sulfuric acid (TNBS) was added to reaction mix which incubated at 50°C for 1 hr. Isolated from light then reaction stopped by adding 0.1 molar Hcl and were left to cool to 30±2°C for 30 min. The absorbance was measured at 340 nm. The degree of hydrolysis (peptides concentration or Peripheral NH3 groups) was estimated by entering the equation below Referred to Jamdar *et al.*, (2010) using UV- PROBE 2.1 program by shimadzu device SP:

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

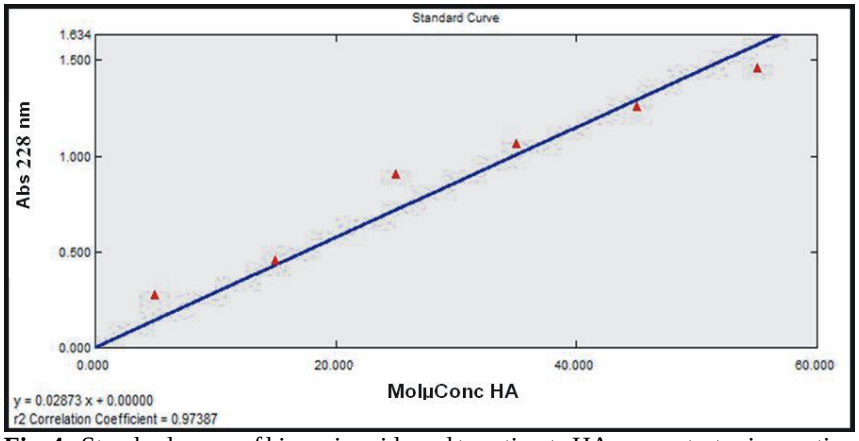


Fig. 4: Standard curve of hippuric acid used to estimate HA concentrates in reaction mixture. The absorbance at 228 nm.

So that

L_t = concentration of Peripheral NH3 groups at time (0-8 hr.).

L_0 = Free amino acids (NH3 group) in original protein samples without any treatment.

$L_{MAX=total}$

L_{MAX} = Total free amino acids resulting from acidic hydrolysis by 6 molar Hcl at 120cp for 24 hr.

Determination of ACE1 Inhibition Activity by α -Casein Hydrolysates.

The efficacy of α_{s2}-CN hydrolysates at each hydrolysis degree in ACE1 inhibition was estimated by spectroscopic method based on the conversion of N-Hippuryl-His-Leu hydrate peptide to Hippuric acid (Benzoylaminoacetic acid) as indicated by ACE1 according to Cushman and Cheung, (1971), modificated by (Chen *et al.*, 2018; Lee *et al.*, 2005).

Volume of 100 µl of α_{s2}-CN hydrolysate was incubated for three periods (beginning, middle and end) of enzymatic hydrolysis for pepsin and trypsin and 1:1 mixture of the two enzymes with 200 µl of Substrate HHL solution at 37°C for 5 minutes using water bath. 20 µL of ACE1 solution 0.2 unit/ ml was added and the reaction mixture incubation at the same temperature for 30 minutes and the enzymatic reaction was stopped using 0.25 ml HCl 1 molar. The amount of hippuric acid produced from the reaction was extracted by adding 3 ml of ethyl acetate at 100°C using a rotary evaporator. 3 ml of distilled water was added to the remaining volume. The absorbance of hippuric acid determined at 228 nm the quantities of HA acid released from HHL estimated according on the standard curve of hippuric acid fig. 3 which was read in the same apparatus. The amount of HA released in the absence of proteolytic enzyme was also considered as 100% ACE1 and the equation (A-B) / A × 100% was used to calculate the enzymatic activity as a percentage: % inhibition = ((B-A) / A) × 100:

A = concentration of hippuric acid = 234.036 µmol, in the absence of any factor that inhibits the effectiveness of ACE1 (100% effectiveness)

B = concentration of hippuric acid in the presence of ACE1 inhibitors, which are peptides under study.

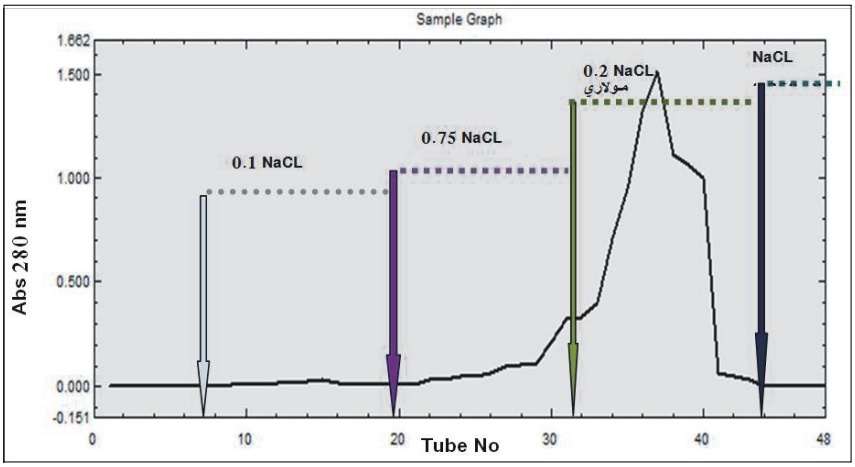


Fig. 5: Purification of α_S-CN of goat milk on the negative ion exchange column DAEA-Cellulose with dimensions of 2×50 cm using a phosphate buffer 0.1 molar pH 7.4 containing urea solution 3.3 mL and 10 mmol mercaptoethanol and runoff 50 ml / h at a rate of 5 ml / tube and salty gradient from 0.1 molar to 0.25 molar sodium chloride in the buffer.

Results and Discussion

Acidic casein yield

The results showed that the percentage of casein obtained after acidic Sedimentation of Iraqi goat milk 2.6%. Very similar to that found by Yangilar, (2013), which set a minimum protein content in goat milk for a number of breeds (3.4%) and total casein (2.4%). The highest protein content in Spanish goat milk is 4.6%, determined by Park *et al.*, (2007). The variance in casein content due to genetic differences between species as well as other such as nutrition, the number of births and other factors that lead to changes in total protein ratios.

Primary α_S- Casein Purification

Method of Zittle *et al.*, (1959) was followed up to separate α_S-CN before purification by chromatographic methods. The yield of α_S-CN by using method of precipitation with salts and urea was 9.2 gm produced from 40gm of acidic casein. This quantity forms 23% and this results are compatible with the amount of α_S-CN obtained by Zittle *et al.*, (1959) when using 30 g of casein to be the result of αs-CN is 7gm, which constitutes a rate of 23.3% CN is possible when the amount of acidic casein increases, but the increase is not 100% straight, It's also compatible with Bramanti *et al.*, (2003) in the rate of 22.4%.

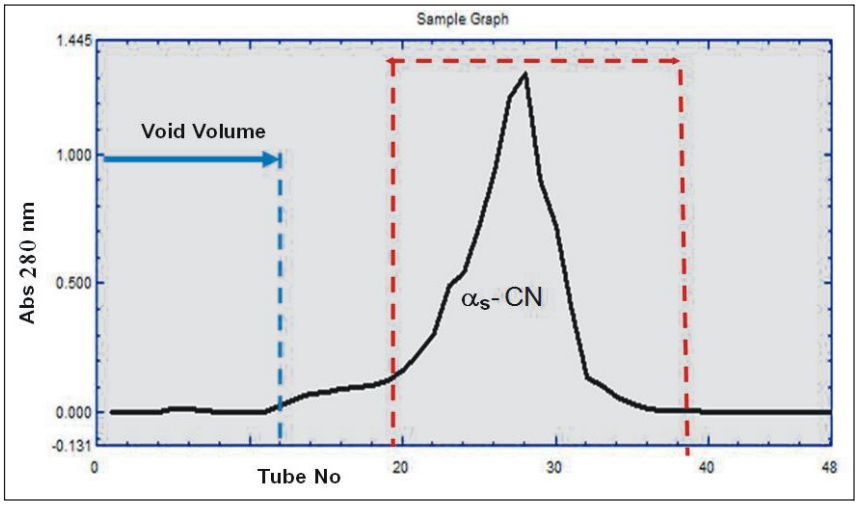


Fig. 6: Final Purification of α_S-CN goat milk using sephadex column G-75 column with dimensions of 90 × 1.5 cm and phosphate buffer solution 0.005 molar with pH 7.6 containing urea 6.6 molar and EDTA 0.004 molar with 20 ml / h flow rate, adjust fraction collector to collect 3 ml volume per tube.

Purification of α_S -Casein by Ion Exchange Method

The first step of the purification process was aimed at eliminating the proteins associated with the α_S-casein by using Sodium chloride salt solution with different molar concentrations of 0.1, 0.175, 0.2 and 0.275 molar, depending on the and α_{S1}-CN, if present in very small quantities, can be separated from α_{S2}-CN, which is present in large quantities in goat's milk, especially if α_S-CN and β-CN is crudely used when the urea solution and mercaptoethanol which contribute to increasing the solubility of the protein and decoding bi-sulfur bonds link (Al-Saadi, 2001). The Wei and Whitney, (1985) and Whitney, (1988) methods were used for the initial purification of α_S-CN from the initial separated goat milk acid casein by push through on the DEAE-Cellulose column and to recover it and disengage from this exchanger, different salt concentrations of NaCl were used. Total casein contains many types of casein, α, β, and κ-CN, each protein differs from the other parts in its negative net charge therefore recovered from DEAE-Cellulose ion exchange column by using concentrations of sodium chloride solution from 0-0.1-0.175-0.2-0.25. fig. 4 shows first peak in the tubes (6-10). It is believed to have been traced back to κ-CN because it is least negative casein and has weak bonding power to the ion exchanger, another peak of β-CN appeared in tubes (22-26) when using NaCl 0.175 M because it has net charge stronger than κ-CN and weaker than α_S-CN. Also the figure shows protein α_S-CN in major peak that appeared in the tubes (32-42)

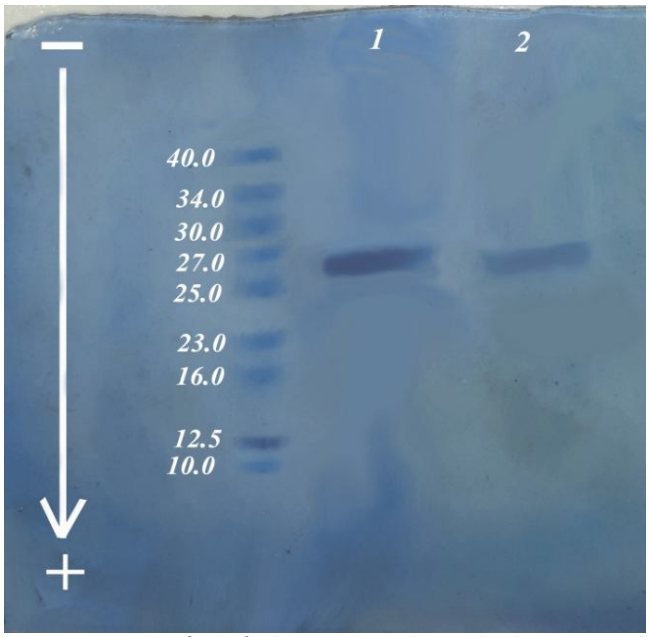
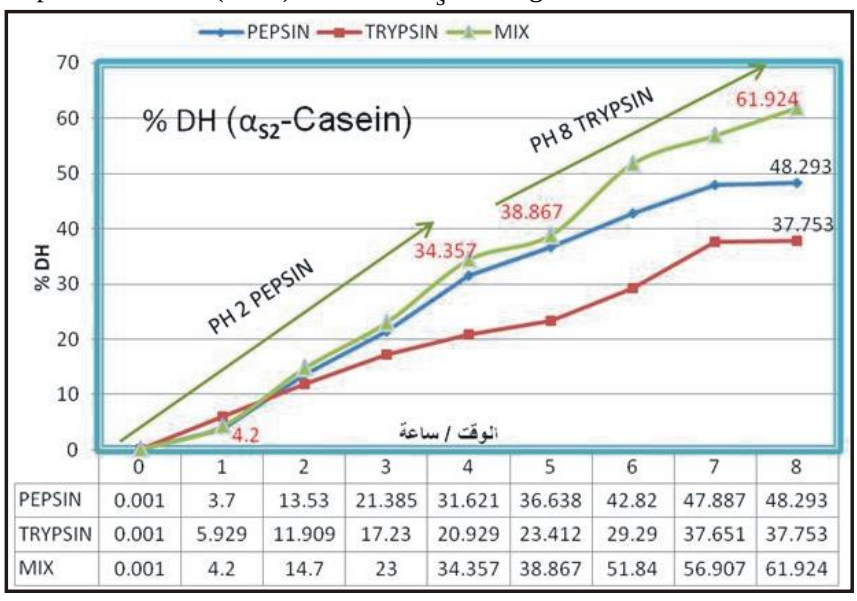


Fig. 7: Migratr of purified proteins with standard protein and standard ladder with a range of 10-40KD in 12.5% SDS-PAGE. 1: represents the standard α_S-CN and No. 2: represents the α_S2-CN purified goat's

because it is carrying negative charge stronger than other protein fractions so that it recovered from the column when using NaCl 0.2 molar. These results are consistent with those obtained by Wei and Whitney, (1985) and Greppi *et al.,* (2008) which indicated the separation of κ-CN in concentrations of less than 0.1 molar sodium chloride due to the low charge of this protein compared with other caseins as Saadi, (2018) confirmed weakness binding of κ-CN protein to negative ion exchange column as the weakest casein in both goat milk and cows. Ruprichova *et al.,* (2015) noted that α_S-CN in goat milk; cows and sheep can be separated by using high salt when using HPLC. The total amount of α_S-CN protein recovered was dried in a freeze-dried method for subsequent purification and the weight obtained was 910, 210 mg of 1 g weight in tubes (32-42), protein concentration rate was 16.569 mg / ml.



	0	1	2	3	4	5	6	7	8
PEPSIN	0.001	3.7	13.53	21.385	31.621	36.638	42.82	47.887	48.293
TRYPSIN	0.001	5.929	11.909	17.23	20.929	23.412	29.29	37.651	37.753
MIX	0.001	4.2	14.7	23	34.357	38.867	51.84	56.907	61.924

Fig. 8: Hydrolysis Percentage of α_S2-Casein induced by pepsin and trypsin and their mixture at a ratio of 1: 1 during incubation for 8 hours on 37°C.

Final Purification of α_S-CN by Gel Filtration Method by Using Sephadex G-75.

Casein contains many proteins, α_S, β and κ-CN, α_S-CN is the largest of these parts. Although the ion exchange step may be enough to purify this protein, additional purification processes achieve a high degree of purity this is what Dosh, (2007) pointed out when she obtained high purity proteins using ion exchange and gel-filtration techniques, so this protein can be purified to a large extent by gel filtration. Fig. 4, shows appearance of a single major peak was in tubes (19-38) and these tubes belong to α_S-CN. This result compatible with Ruprichova *et al.,* (2015) and Vincenzetti *et al.,* (2005) suggesting that the main peaks of the parts of the cows, sheep and goat milk cows are ion-exchanged and then gel-filtrated with the sephadex G-25 column or using RP-HPLC.

These results are in compatible with al-Saadi, (2001), when he obtained α_S-CN by ion exchange method only without resorting to gel filtration and proved the purity of this protein later when conducting the electrophoresis of this protein in the gel acrylamide when he appeared in one band. Weights averaged 401.43 mg of 500 mg used at the beginning of the purification process and the protein concentration rate was 8.36 mg / ml. α_S-CN was then concentrated, freeze dried and kept at freezing temperature.

Confirm the Purity and Molecular Weight of α_S-CN by SDS-PAGE Method

Fig. 6, shows the appearance of single-purified α_{S2}-CN band, confirming that it is free from interference other casein fractions; having traveled approximately the same distance as standard proteins this confirms the purity of protein. α_{S2}-CN is the predominant in goat's milk casein, as indicated by Moioli *et al.,* (1998) and Anema *et al.,* (1998). In contrast to cow's milk. It follows that the milk of this type of goat does not contain or contains very little α_{S1}- CN protein. Compared with the molecular weight of the corresponding protein in Ladder, the molecular weight of pure α_{S2}-CN was estimated about 25.620 KD.

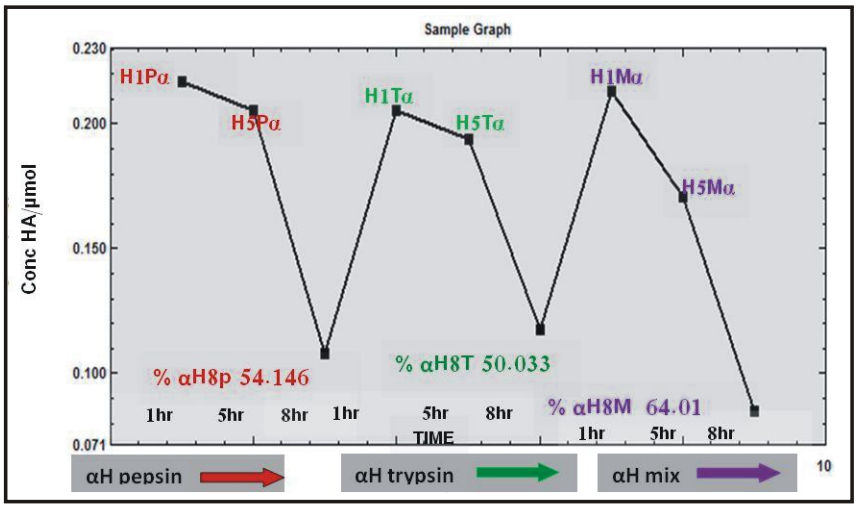


Fig. 9: Inhibition of angiotensin-converting enzyme 1 (ACE1) by α_S2-CN hydrolysates (Hα) induced by pepsin and trypsin and their 1: 1 mixture.

This molecular weight corresponds to what Raak *et al.*, (2018) found when SDS-PAGE casein was migrated and found it to be less than 26.8 KD. The differences in the molecular weights of the fractions of the caseins in the milk of different species are mainly due to genetic factors.

Hydrolysis by Enzymes Pepsin and Trypsin and Hydrolysis by Pepsin with Trypsin for α_{S2} Casein.

Hydrolysis of α_{S2}-CN casein was carried out using pepsin and trypsin, both of which were combined at a ratio of 1:1 for 8 hours. The results show in fig. 7 that the degree of hydrolysis DH increases with the incubation time of the two enzymes and their mixture. This is evidenced by the high absorption values along the wavelength of 340 nm this means increased release of peptides resulting from the specific hydrolysis action of the enzyme and the overall behavior of the three treatments is similar in that they reached the maximum hydrolysis after 8 hours of incubation at 37°C but in different proportions 48.293, 37.753, 61.924% with Pepsin and trypsin and their mixture respectively. These ratios are consistent with Lee *et al.*, (2005) when treated of acidic casein from goat milk with multiple enzymes, including pepsin and trypsin indicates the availability of cyclic amino acids that form the alkaline for action of pepsin enzyme in α_{S2} protein. As a result of the synergistic action of the two enzymes together on the DH of α_{S2}-CN, the results indicated in fig. 7, that there is a clear increase in the values of DH was after 4 hours of incubation with pepsin enzyme is 34.357% and increased after incubation with trypsin another 4 hours to 61.924.

This result is consistent with Sun *et al.*, (2014) when using these enzymes together to hydrolyze α_S-CN and concluded that synergistic action of enzyme contributes to obtain a high degree of hydrolysis as the initial hydrolysis by the action of pepsin enzyme transforms the complex protein form into a free polypeptide due to the fact that the action of the enzyme pepsin is specialized in attacking peptide bonds due to the most Cyclic amino acids Tyr Trp and Phe as well as less hydrophobic amino acids and make the peptide chain more open which contributes to making the amino acid Arg and Lys more vulnerable and more ready to be attacked by trypsin.

Effectiveness ACE_1 Inhibitory of α_{S2}-CN (αH) Hydrolysates

Fig. 8, shows the effect of hydrolysis of three time periods resulting from the action of pepsin enzyme in α_{s2}-CN resulting after 1, 5 and 8 hours of incubation with ACE1 (αH1P, αH5P and αH8P) as well as three hydro of the same previous readings but by the enzyme trypsin in αH1T, αH5T and αH8T and their indicated mixture αH1M αH5M and αH8M on the effectiveness of ACE1, which is the concentration of hippuric acid HA released by the activity of ACE1 in substrate Hippuryl histidine leucine HHL. The diagram 8 shows also an inverse proportion between the HA concentration and the inhibition percentage and the inhibition rate was higher than 50% for the hydrolysates produced after 8 hours of 54.146, 50.033 and 64.019% by pepsin and trypsin at a ratio of 1:1 and their mixture respectively at a

Table 1: Relationship of DH ratio and values of α_{S2}-CN Hydrolysis concentrations with HA concentration and ACE1 inhibition efficacy.

Hydrolysates $_{S2}$-CNα	H1pα after 1hr	H5pα after 5hr	H8pα after 8hr	H1Tα after 1hr	H5Tα after 5hr	H8Tα after 8hr	H1Mα after 1hr	H5Mα after 5hr	H8Mα* after 8hr
Inhibition activity %	7.251	12.187	54.146	12.187	17.123	50.033	8.896	26.996	64.019*
Hippuric acid Conc	0.217	00.205	00.107	00.213	00.171	0.117	0.205	0.194	00.084
Hydrolysis degree %	3.700	36.638	48.293	05.929	23.412	37.753	4.200	38.860	61.924
Peptides Conc (group NH_3)	3.023	29.794	39.267	04.835	19.044	30.700	3.66	31.600	50.340
Highest ACE1 inhibition ratio of S2-CNα hydrolysates: *									

concentration of 0.117 HA, 0.084 and 0.107µmol (Table 1). This means that DH led to increased inhibitory activity of the released hydrolysates, whether only peptides group or a single peptide chain, all of which were released from α_{S2}-CN. From the table 1, the increase in the inhibitory activity was noticeable between 5 hours to the end of the incubation of 8 hours while the rise was between 1 hour and 5 hours less. The hydrolysates with the highest inhibitory activity was αH8M of 64.019% with a concentration of peptides 50.346 mMol.

Bioactive peptides block the process of HHL conversion to HA and this theory has been confirmed by most studies conducted to demonstrate the effect of protein decomposers on the effectiveness of ACE1 one of the results of Sun *et al.*, (2014) when hydrolyzing casein with pepsin and trypsin to obtain an inhibitory efficiency of 72% after 6 hours and the concentration of peptides 60.06 mMol. It is also consistent with Lee *et al.*, (2005), which received a 96% inhibition in pepsin and 67% in trypsin only when the DH value of goat milk casein hydrolyzed to more than 48%. On the other hand, some researchers pointed out that the rise in the degree of hydrolysis is not the only factor leading to high efficiency inhibition of ACE1 Munn, (2013). The effect of high hydrolysis on the inhibitory efficiency (especially low inhibition at the beginning of the degradation process) is explained by the high concentration of peptides (quantitatively) and the increased chances of their diversification, especially when more than one type of enzyme is used and thus correlates with ACE1 catalytic sites and blocking the enzyme union With the substrate on which the enzyme acts *in vivo* (Chiang *et al.*, 2006).

Conclusion

Local goat milk protein hydrolysates, especially α_{S2}-CN, have the ability to inhibit ACE1 in vitro efficiently when using pepsin or trypsin and mix of them by ratio of 1:1 because these hydrolysates contain low molecular weight ACE1 inhibition peptides. Separating these peptides from their hydrolysates may increase their effectiveness. Peptides from other goat milk casein fractions hydrolysates (β, κ-CN) may also have ACE1 inhibition effectiveness. *In vivo* studies to choose the effective hydrolysate which lowering blood pressure are important to establish the importance of eating goat milk and its products in lowering blood pressure because protein will be digested by digestive enzymes in the gut and release antihypertensive peptides.

References

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.

Al-Saadi, J.M. (2018). Studying afficaicy of three purification Methods of Local Milk Goat κ-CN. *Journal of Kerbala for Agricultural Sciences.*, **5 (5):** 100-107.

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.

Aslam, M.Z., S. Shoukat, H. Zhao and B. Zhang (2018). Proteomic Analysis of ACE Inhibitory Peptides extracted from Fermented Goat Milk. *bio. Rxiv.*, 336107.

Aluko, R.E. (2012). Functional foods and nutraceuticals (p. 155). New York: Springer.

Aluko, R.E. (2018). Antihypertensive Foods: Protein Hydrolysates and Peptides.

Brown, B. and A.S. Hall (2005). Renin-angiotensin system modulation: the weight of evidence. *American journal of hypertension.*, **18(S5):** 127S-133S.

Bansal, N. and B. Bhandari (2016). Functional milk proteins: Production and utilization-Whey-based ingredients. In *Advanced dairy chemistry.*, (67-98). Springer, New York, NY.

Bramanti, E., C. Sortino, M. Onor, F. Beni and G. Raspi (2003). Separation and determination of denatured α_{S1}-, α_{S2}-, β- and κ-caseins by hydrophobic interaction chromatography in cow's, ewe's and goat's milk, milk mixtures and cheeses. *Journal of Chromatography A.*, **994(1-2):** 59-74.

Costa, E.L., A.R. Almeida, F.M. Netto and J.A.R. Gontijo (2005). Effect of intraperitoneally administered hydrolyzed whey protein on blood pressure and renal sodium handling in awake spontaneously hypertensive rats. *Brazilian journal of medical and biological research.*, **38(12):** 1817-1824.

Cushman, D.W. and H.S. Cheung (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochemical pharmacology.*, **20(7):** 1637-1648.

Chiang, W.D., M.J. Tsou, Z.Y. Tsai and T.C. Tsai (2006). Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor. *Food Chemistry.*, **98(4):** 725-732.

Chen, L., J. Wang, G. Shu and H. Chen (2018a). Production of Angiotensin-I-Converting Enzyme inhibitory peptide from goat milk casein: optimization conditions of complex protease hydrolysate by response surface methodology and purification. *Emirates Journal of Food and Agriculture.*

Chen, Li, Qiuhong Zhang, Zhe Ji, Guowei Shu and He Chen (2018). "Production and fermentation characteristics of angiotensin-I-converting enzyme inhibitory peptides of goat milk fermented by a novel wild Lactobacillus plantarum 69." *LWT* **91:** 532-540.

Chen, H., Z. Ji, G.W. Shu and H.N. Xing (2012). Effect of probiotic Lactobacillus strains on angiotensin I converting enzyme inhibitory activity from fermented goat milk. In *Advanced Materials Research* (**531:** 442-445). Trans Tech Publications.

Dosh, Kifah Said Abbas (2007). Ph.D. thesis. College of Agriculture / University of Baghdad. Purification and characterization of some polymorphic white blood cell proteins isolated from cow's milk with mastitis and its role in ripening cheddar cheese.

Guyer, A.C. and A. Banerji (2015). ACE inhibitor-induced angioedema. *UpToDate*.

Greppi, G.F., P. Roncada and R. Fortin (2008). Protein components of goat's milk. *Dairy goats feeding and nutrition.*, **2:** 71-94.

Jamdar, S.N., V. Rajalakshmi, M.D. Pednekar, F. Juan, V. Yardi and A. Sharma (2010). Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food Chemistry.*, **121(1):** 178-18.

Khafaji, Zahra Mahmoud (2008). Chapter Four. Therapeutic Biology, 153-157.

Korhonen, H. and A. Pihlanto (2007). Technological options for the production of health-promoting proteins and peptides derived from milk and colostrum. *Current pharmaceutical design.*, **13(8):** 829-843.

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.

Laragh, J. (2001). Laragh's lessons in pathophysiology and clinical pearls for treating hypertension. *American journal of hypertension.*, **14(9):** 837-854.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature.*, **227(5259):** 680.

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.

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.

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.

Munn, S.M. (2013). Dairy and Soy Derived Bioactive Peptide and the Renin-Angiotensin-Aldosterone System. A thesis submitted to the faculty of the graduate school of the university of Minnesota for the degree of master of science.

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.

Park, Y.W., M. Juarez, M. Ramos and G.F.W. Haenlein (2007). Physico-chemical characteristics of goat and sheep milk. *Small ruminant research.*, **68(1-2):** 88-113.

Rutella, G.S., L. Solieri, S. Martini and D. Tagliazucchi (2016). Release of the antihypertensive tripeptides valine-proline-proline and isoleucine-proline-proline from bovine milk caseins during *in vitro* gastrointestinal digestion. *Journal of agricultural and food.*

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)**.

Raak, N., R. Abbate, A. Lederer, H. Rohm and D. Jaros (2018). Size separation techniques for the characterisation of cross-linked casein: A review of methods and their applications. *Separations.*, **5(1):** 14.

Shi, A., H. Liu, L. Liu, H. Hu, Q. Wang and B. Adhikari (2014). Isolation, purification and molecular mechanism of a peanut protein-derived ACE-inhibitory peptide. *PloS one.*, **9(10):** p.e111188.

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.

Schägger, H. (2006). Tricine-SDS-PAGE. *Nature protocols.*, **1(1):** 16.

Saadi, Jassim Mohammed Saleh (2001). Master Thesis. Study of goat milk and cow proteins and their relationship to hypersensitivity.

Sica, D.A. (2005). Angiotensin Converting Enzyme Inhibitors' Side Effects-Physiologic and Non Physiologic Considerations. *The J. of Clinical Hypertension.*, **7:** 17-23.

Vincenzetti, S., P. Polidori, E. Salimei, P.L. Mariani, S. Allegri, N. Cammertoni, B. Quadrini and A. Vita (2005). Purification and Identification of α_{S1} and β-Caseins from Asses Milk. *Veterinary research communications.*, **29(2):** 211-213.

Whitney, R.M. (1988). Proteins of milk. In *Fundamentals of dairy chemistry* (81-169). Springer, Boston, MA.

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.

Wong, J., R.A. Patel and P.R. Kowey (2004). The clinical use of angiotensin-converting enzyme inhibitors. *Progress in cardiovascular diseases.*, **47(2):** 116-130.

Yangilar, F. (2013). As a potentially functional food: goats' milk and products. *J. Food Nutr. Res.*, **1(4):** 68-81.

Zittle, C.A., J. Cerbulis, L. Pepper and E.S. Della Monica (1959). Preparation of calcium-sensitive α-casein. *Journal of Dairy Science.*, **42(12):** 1897-1902.