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Comparative Antioxidant Properties of Buffalo and Bovine Casein Hydrolysates

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***Abstract* – The aim of this work was to investigate the enzymatic hydrolysis of whole casein from buffalo milk with proteolytic enzymes, alcalase, and typsin, and to assess the antioxidant activity of hydrolysates. Antioxidant of casein hydolysate was separated and purified using ultrafiltration followed gel filtration and antioxidant peptides were determined. Degree of hydrolysis of alcalase hydrolysis was higher than trypsin hydrolysis for both of buffalo and bovine casein(92.26 and 86.43%) respectively after 2h hydrolysis. By Sephadex G-15 gel chromatograms, Fraction 2 and 3from bovine casein hydrolysates by trypsin were confirmed the highest antioxidant activity (92.54 and 92.59%) respectively. Also Fraction 2 from alcalase hydrolysates was the highest in antioxidant activity. The most abundant amino acid was Isoleucine and prolinein buffalo casein. Based on the results of this experiment, buffalo and bovine casein hydrolysae by trypsin were shown to have antioxidant activity and provide beneficial antioxidant properties in functional foods and pharmaceuticals.**

***Keywords* – Buffalo Casein, Bovine Casein, Amino Acids, Hydrolysate, Antioxidant Activity.**

I. INTRODUCTION

Recently, the novel usage of casein serves as an excellent source of bioactive peptides and related ingredients such as its enzymatic hydrolysates and peptides has attracted increasing attention [1]. Additionally, various sources of casein bioactive peptides were previously produced from different casein animal species bovine milk [2], yak milk [3], goat milk [4], and buffalo milk [5]. Casein-derived peptides have been shown to have a range of effects, such as antihypertensive, antithrombotic, antimicrobial, opioid, immune-modulating, and mineral binding [6, 7, 8]. Casein is also an important source of antioxidant peptides, and multiple studies have shown that casein hydrolysates can be used as peptides for therapeutic purposes and can be found in dairy foods [9]. The majority of antioxidant peptides derived from food sources were consisted by 2–16 amino acids with the molecular weight ranging from 500 to 3000 [10]. Besides, the demand for the use of peptides and proteins as antioxidants in food is increasing due to the low costs, safety and their inherent nutritional and functional values [11]. Buffalo milk is ranked at second after cow milk, based on worldwide milk production and distribution with leading countries in Asia (India, Pakistan and China) and the Middle East (Egypt) and Europe. Notably, about 103 million tons of buffalo milk was produced in 2013, representing 13% of the total world milk production with an annual growth rate of ~3.3% which is higher than cow milk (annual growth rate 0.9%) [12]. Besides, if compared with cow's milk, buffaloes' milk has a protein content in the range of 3.8-4.5% with a high casein ratio[13, 14]. Antioxidant peptides can act as radical scavengers. Therefore, it is important to inhibit oxidation reactions and formation of free radicals in food products and the living body [15]. Rival et al. (2001) [16] concluded that casein-derived peptides, as preferred target over fatty acid radicals, inhibited enzymatic and non- enzymatic lipid peroxidation. This peptides were obtained by the enzymatic hydrolysis of casein had antioxidant activity [2, 3]. Trypsin hydrolysis was able to improve the scavenging activity of both the isolate and the protein fractions by causing the release of small peptides and/or free amino acids with such activity [15]. In addition, casein trypsin digests showed inhibitory properties in the oxidation of linoleate by lipoxygenase, peroxyl radicals and 2,20-azinobis-(3-ethylbenzothiazoline- 6-sulphonic acid (ABTS) radical [16]. Korhonen and Pihlanto (2006) [17] reported that the most common way to produce bioactive peptides is proteolytic enzymes such as pepsin, trypsin, alcalase, and pancreatin [18]. Wang, Jia, and Yang, (2013) [19] mentioned that, several studies have been made in the production of casein hydrolysates and casein bioactive peptides, microstructure characteristics and particle size distribution of casein hydrolysates obtained by enzymatic hydrolysis, the role in functional properties of casein hydrolysates [19]. Much work regarding those peptides, which are known to possess bioactivities, is currently underway regarding their release via selective enzymatic hydrolysis[20].Buffalo casein is less studied than the bovine casein. To our knowledge, naturally occurring peptides from buffalo casein has not been reported yet. The information on characterize and compare buffalo and bovine casein which naturally occurring peptides, that information about buffalo casein needs to be improved. The objective of this study was to evaluate and compare the antioxidant properties of buffalo and bovine casein hydrolysate.

II. MATERIAL AND METHOD

Materials

Sodium caseinate bovine milk (CN) was purchased from Tokyo chemical industry Co., LTD. (Tokyo, Japan). Trypsin (EC no 3.4.21.4, pH 7–9 at 37 °C) and alcalase (EC

no of 3.4.21.62, pH 5 – 7 at 55 °C) were purchased from Novo Enzyme (Bagsvaerd, Denmark). Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were procured from Sinopharm chemical reagent Co., Ltd, Shanghai, China. All solutions were prepared in distilled water.

Preparation of Sodium Caseinate from Buffalo Milk (CB)

Skim milk was prepared from fresh whole buffalo milk obtained from the Farm of Faculty of Agriculture, (Cairo University, Cairo, Egypt) by centrifugation (Milk centrifuge Elecrem, Helmut Rink GmbH, Amtzell, Germany) at 2000 ×g for 30 min at 4 °C. The average composition of skim milk in dry weight was 10.62, 0.1, 4.23, 4.92, and 0.97% for total solids, fat, protein, lactose and ash respectively.

Sodium caseinate from buffalo milk was prepared according to the method of Mulvihill (1992)[21] with some modifications. Skim milk was acidified to pH 4.6 with hydrochloric acid (1 M) under continuous stirring by stirrer (IKA@-works guangzhou Co., Guangzhou, China) at 25 °C. After leaving of curd deposition for 20 min, the mixture was filtered (Whatman no. 40). The precipitated casein was washed with distilled water, then dissolved with the addition of NaOH (1 M) at pH 7.0, and again left for precipitation. Precipitation and washing steps were repeated four times. The final precipitates were dissolved in NaOH (1 M) to pH 7.0, thereafter heated at 80 °C for 30 min to inactivate plasmin, dialysed against distilled water and lyophilized.

III. Chemical Analysis

Determination of Total Amino Acid Compositions

Amino acid compositions of casein were determined as described by Adeyeye et al. (2009) [22] with slight modifications. Briefly, 30 mg of casein sample was poured into glass ampoules, and 7 mL of 6-M HCl was added and oxygen was expelled by bubbling with nitrogen. Glass ampoules were sealed with a flame and were then heated at 105 ± 5 °C for 22 h. Ampoules were then cooled and opened and the contents were filtered. Filtrates were evaporated to dryness at 40 °C under a vacuum. Residues were dissolved in 5-mL aliquots of acetate buffer (pH 2.0), and samples (1 µL) were injected into a Zorbax 80 A C_{18} column (4.6 × 180 mm, Agilent Technologies) at 40 °C with detection at 338 nm. Mobile phase A comprised 7.35-mM sodium acetate/triethylamine/tetrahydrofuran (500:0.12:2.5, v/v/v) and was adjusted to pH 7.2 with acetic acid, and mobile phase B (pH 7.2) comprised 7.35-mM sodium acetate/methanol/acetonitrile (1:2:2, v/v/v). Amino acid compositions were expressed as g of amino acid per 100 g of protein. Amino acids contents were calculated according to peak areas relative to those of standards. Tryptophan contents were determined using Alkaline hydrolysis

Preparation of Casein Hydrolysates

Casein solution (2% w/w on protein basis) was prepared by dissolving ~2.2 g of casein powder (according to percent of protein in casein powder) in 100 g distilled water; dispersion was stirred for 1 h at room temperature and kept overnight at 5 ° C. On the next day, 80 mg/mL enzyme solution (dissolved in distilled water) was added to make the hydrolysates. The pH and temperature of the casein solution was adjusted according to the characteristics of the enzymes as described above. The enzymes were pre-incubated for 10 min and the enzyme to substrate ratio 1:100 (w/w) was used in all experiments. The hydrolysis process was started by the addition of the enzyme solution to the casein solution with 30 s vortexes. The reaction mixture was quenched after 3 h of hydrolysis, then heated at 90 °C for 15 min to stop the enzyme activity followed by cooling (20 min in ice bath) and centrifugation (Model 3K16, Sigma Labor zentrifugen GmbH, Osterode am Harz, Germany) (10 min at 12,000 ×g).

Enzymatic Hydrolysis and Degrees of Hydrolysis (DH)

The titrant used in this procedure was NaOH and exact normality of fresh batches of about 0.02-N NaOH was standardized (in triplicate) against a known concentration of HCl. DH of proteins were calculated according to Lemos, Lawrence, and Siccardi(2009) with some modifications as follows:

$$DH = B \times N_B \times {}^{1}/_{\alpha} \times {}^{1}/_{M_p} \times {}^{1}/_{h_{tot}} \times 100$$

Where B = consumption of NaOH during hydrolysis (mL) − consumption of NaOH in the blank (mL), N_B is the normality of NaOH titrant s(mol/L), α is the average degree of dissociation of α–NH groups, 1/α is 1.2 at 40 °C and pH 8.0, M_p is the total mass of protein (g) in the reaction mixture from test ingredient and added enzyme, and h_{tot} is the total number of peptide bonds in casein (8.2 meqv/g protein; [23]).

Separation and Purification of Antioxidant Peptides

The obtained hydrolysate was subjected to fractionation by ultra filtration with 1kDa MWCO (polyethersulfone membrane, Sartorius Stedim Biotech, Aubagne, France). The fraction 1k Dawas further analyzed by purification of antioxidant peptides in hydrolysates adopted the method of Maruyama et al. (1985)[24]. Gel filtration chromatography was performed on Sephadex G-15 (Sigma Co., USA). The column (2.5×50 cm) previously equilibrated with the distilled water, operated at a flow rate of 0.2 ml min-1 and fraction of 2 ml were collected and analyzed by UV-absorbance (Perkin Elmer Lambda EZ 201, USA) at 280 nm. The samples were filtered through 0.5 m syringe filters prior to application to the column. The fraction with the highest antioxidant activity was further analyzed by reverse phase high performance liquid chromatography (RP-HPLC)

Determination of ABTS Radical Scavenging Activity

Radical scavenging activity was tested using the method of Hernández-Ledesma, Dávalos, Bartolomé & Amigo (2005) [25] with some modification. ABTS radical cation ($ABTS^+$) was prepared 12–16 h before using into a 25 mL volumetric flask by dissolving 7 mM ABTS stock solution with 2.45mM potassium persulfate in distilled water and the solution was kept at room temperature in the absence of light. The $ABTS^+$ solution was diluted in 5mM phosphate buffer saline (PBS, pH 7.4), and used for setting absorption at 734 nm in a 3 cm cuvette until (0.70 ±0.02) absorbance value was achieved at 30°C. Standard curve was obtained by adding 2 mL of diluted $ABTS^+$ solution to 20µL of

Trolox with a final concentration of Trolox ranging from 0 to 8 mg/mL in PBS. The absorbance reading was taken after 10 min at 30 °C. Appropriate blank solvent was run in each assay. Activity of each 20 µL sample was measured in triplicate. The percent of inhibition was calculated as a function of antioxidant concentration corresponding to Trolox as a standard compound. To calculate the Trolox Equivalent Antioxidant Capacity (TEAC), the gradient of the plot of the percentage inhibition of absorbance sample concentration was divided by the gradient of the plot for Trolox. This gives the TEAC at a specific time.

Statistical Analysis

Data for each treatment condition are presented as mean ± standard deviation. One-way ANOVA was conducted using SPSS 19 (SPSS Inc., Chicago, USA) with significance defined as the 95% confidence limit (P<0.05).

IV. Result and Discussion

Amino Acid Compositions

Amino acids contents in CB and CN in Table 1. Differing hydrophobic (Leu, Val, and Phe), hydrophilic (Asp, Ser, Arg, and Glu), basic (His, Pro and Lys), and aromatic amino acids (Phe and Tyr) may contribute to differences in antioxidant activities (Najafian et al., 2015). Accordingly, amino acid compositions of CB and CN differed, and the relative predominance some amino acids likely provided electron donors with efficient antioxidant activity (Kumar et al., 2013). CN had higher contents of His, Tyr, Val, Phe, and Lys than CB. Conversely, CB had a higher proportion of hydrophobic amino acids (58.01%).

Degrees of Hydrolysis (DH)

Degree of hydrolysis (DH) was determined by titration of hydrolsate by NaOH 1 M in these hydrolysate. Two types of CB and CN were assessed and DH by the two different enzymes trypsin and alcalase are shown in Table 2. Differences in DH can be attributed to enzyme cleavage specificity and the numbers of cleavage sites located in protein chains of casein [26].The higher the DH, the higher the content of released amino groups. Degree of hydrolysis found maximum with alcalase treatment. This shows that alcalase utilized more protein as substrate to cause hydrolysis as compared to trypsin. Treatment with trypsin yields minimum DH indicating that buffalo casein is most resistant to this enzyme. Treatment of buffalo casein with alcalase for 120 min shows highest hydrolysis. The same result of hydrolysis obtained when treated with trypsin. It shows that degree of hydrolysis increases with the time of incubation[3] also malcalase ant trypsin hydrolyzed showed CN was slightly lower than those for CB during 2h hydrolysis.

Antioxidant Activity of Buffalo and Bovin Casein

The fraction of 1k Daultrfiltration alcalase and trypsin treated was further separated by sephadex 15gel chromatograpmy as shown in Fig. 1. Alcalase and trypsin was showed four and three fraction for 1k Da UF CN and CB respectively. Each peak was collected and measured for the antioxidant activity by ABTS (Fig. 1 (A), (B), (C) and (D).

Based on the mechanism of gel filtration, the MW of each fraction was increasingly lower as the elution time increased. Ji, Sun, Zhao, Xiong, and Sun, (2014) [27] reported that gel filtration was applied to isolate the protein hydrolysates according to their molecular weight. Sephadex G-15, with the separation range of <1000was suitable for the separation of peptides 1 kDa UF [28]. Therefore, trypsin hydrolysate was displayed with a higher antioxidant activity after. Among the four peaks, the F2 and F3 exhibited the highest antioxidant activity (92.59% and 92.54% respectively) (P< 0.05). While,F2exhibited the highest antioxidant activity in CB fraction by trypsin (65.34%) but lower than CN fraction. With regard to, Alcalase hydrolysate F2 and F3 showed the highest antioxidant activity for CB(22%) and CN(35%) respectively. Generally, Similar results were reported by other researchers for buffalo casein and sweet potato protein hydrolysates that the highest antioxidant activity was contributed by peptides containing high amount of low MW<1kDa[29].

V. Conclusion

Our results show that antioxidant peptides are applicable in therapeutic formula. Trypsin hydrolysate was high antioxidant peptide produced from buffalo and bovine casein. A future study shall be carried out on the commercialization of the peptides with antioxidant activity by compounding a large amount of such peptides and conducting an in vivo test.

References

[1] Tu, M., Feng, L., Wang, Z., Qiao, M., Shahidi, F., Lu, W., & Du, M. (2017). Sequence analysis and molecular docking of antithrombotic peptides from casein hydrolysate by trypsin digestion. Journal of Functional Foods, 32, 313–323.

[2] Irshad, I., Kanekanian, A., Peters, A., & Masud, T. (2015). Antioxidant activity of bioactive peptides derived from bovine casein hydrolysate fractions. Journal of Food Science and Technology, 52(1), 231–239.

[3] Kumar, S., Chouhan, V. S., Sanghi, A., & Teotia, U. V. S. (2013). Antioxidative effect of yak milk caseinates hydrolyzed with three differenct proteases. Veterinary World, 6(10), 799–802.

[4] Ahmed, A. S., El-Bassiony, T., Elmalt, L. M., & Ibrahim, H. R. (2015). Identification of potent antioxidant bioactive peptides from goat milk proteins. Food Research International, 74, 80–88.

[5] Abdel-Hamid, M., Otte, J., De Gobba, C., Osman, A., & Hamad, E. (2017). Angiotensin I-converting enzyme inhibitory activity and antioxidant capacity of bioactive peptides derived from enzymatic hydrolysis of buffalo milk proteins. International Dairy Journal, 66, 91–98.

[6] Silva, S. V., & Malcata, F. X. (2005). Caseins as source of bioactive peptides. International Dairy Journal, 15,1-15.

[7] Hernández-Ledesma, B., Amigo, L., Ramos, M., & Recio, I. (2004). Application of high performance liquid chromatography-tandem mass spectrometry to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion. Journal of Chromatography A, 1049, 107–114

[8] Phelan, M., Aherne, A., FitzGerald, R. J., & O'Brien, N. M. (2009). Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status. International Dairy Journal, 19(11), 643–654.

[9] Pandya, A. J., & Haenlein, G.F.W. (2009). Bioactive components in buffalo milk. In P. Y. W (Ed.),Bioactive Components in Milk and Dairy Products (pp. 105–158).New York: Wiley

[10] Xing, L. J., Hu, Y. Y., Hu, H. Y., Ge, Q. F., Zhou, G. H., & Zhang, W. G. (2016). Purification and identification of antioxidative peptides from dry-cured Xuanwei ham. Food Chemistry, 194, 951–958.
[11] Vaštag, Ž., Popović, L., Popović, S., Krimer, V., & Peričin, D. (2011). Production of enzymatic hydrolysates with antioxidant and angiotensin-I converting enzyme inhibitory activity from pumpkin oil cake protein isolate. Food Chemistry, 124(4), 1316–1321.
[12] IDF (2014). IDF bulletin NO. 476/2014 Brussels, Belgium International Dairy federation.
[13] Abd El-Salam, M. H., & El-Shibiny, S. (2011). A comprehensive review on the composition and properties of buffalo milk. Dairy Science & Technology, 91(6), 663–699.
[14] Ahmad, S., F.M. Anjum, N. Huma, A. S. and T. Z., & National. (2013). Composition and Physico-Chemical Characteristics of Buffalo Milk With Particular Emphasis on Lipids, Proteins,Minerals, Enzymes and Vitamins. Journal of Animal and Plant Sciences, 23, 62–74
[15] Wattanasiritham, L., Theerakulkait, C., Wickramasekara, S., Maier, C. S., & Stevens, J. F. (2016). Isolation and identification of antioxidant peptides from enzymatically hydrolyzed rice bran protein. Food Chemistry, 192, 156–162.
[16] Rival, S.G., Boeriu, C.G., & Wichers, H.J. (2001). Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition, Journal of Agricultural and Food Chemistry, 49, 295–302.
[17] Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. International Dairy Journal, 16(9), 945–960.
[18] Kim, S. B., Seo, I. S., Khan, M. A., Ki, K. S., Nam, M. S., & Kim, H. S. (2007). Separation of iron-binding protein from whey through enzymatic hydrolysis. International Dairy Journal, 17(6), 625–631.
[19] Wang, J., Jia, F., & Yang, G. (2013). Changes in Particle Size Distribution and Solubilisation of Casein Protein during Enzymatic Hydrolysis. Focusing on Modern Food Industry, 2(4).
[20] Pralea, D., Dumitrascu, L., Borda, D., & Stănciuc, N. (2011). Functional properties of sodium caseinate hydrolysates as affected by the extent of chymotrypsinolysis. Journal of Agroalimentary Processes and Technologies, 17(3), 308–314
[21] Mulvihill, D.M. (1992) Production, functional properties and utilization of milk protein products, in Advanced Dairy Chemistry, Volume I-Proteins, (P.F. Fox ed.) Elsevier Applied Science Publishers, London, pp. 369-404
[22] Adeyeye, E. I. (2009). Amino acid composition of three species of Nigerian fish: *Clariasanguillaris, Oreochromisniloticus* and *Cynoglossus senegalensis*. *Food Chemistry, 113*, 43–46.
[23] Adler-Nissen, J. (1986). Methods in food protein hydrolysis.In Enzymatic hydrolysis of food proteins (pp. 110-130). New York: Elsevier Applied Science Publishers.
[24] Maruyama, S., K. Nakagomi, N. Tomizuka and H. Suzuki. 1985. Angiotensin I-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II. Isolation and bradykinin-potenting activity on the uterus and the ileum of rats. Agriculure. Biology of Chemical. 49:1405.
[25] Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., & Amigo, L. (2005).Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. Journal of Agricultural and Food Chemistry, 53, 588–593.
[26] Najafian, L., & Babji, A. S. (2015).Isolation, purification and identification of three novel antioxidative peptides from patin (*Pangasiussutchi*) myofibrillar protein hydrolysates. *LWT - Food Science and Technology, 60*, 452–461.
[27] Ji, N., Sun, C., Zhao, Y., Xiong, L., & Sun, Q. (2014). Purification and identification of antioxidant peptides from peanut protein isolate hydrolysates using UHR-Q-TOF mass spectrometer. Food Chemistry, 161, 148–154
[28] Lee, K. J., Kim, S. B., Ryu, J. S., Shin, H. S., & Lim, J. W. (2004). Separation and purification of angiotensin converting enzyme inhibitory peptides derived from goat's milk casein hydrolysates. Asian-Australasian Journal of Animal Sciences, 18, 741–746
[29] Shanmugam, V. P., Kapila, S., Sonfack, T. K., &Kapila, R. (2015). Antioxidative peptide derived from enzymatic digestion of buffalo casein. International Dairy Journal, 42(1), 1–5

Table 1. Amino Acid Composition (Grams per 100 g) of Sodium case in Commercial (SCC) , Buffalo (SCB)

Amino Acids	CN	CB
Asp	6.79	5.95
Glu	22.38	21.37
Ser	4.32	4.23
His	2.91	2.47
Gly	1.75	1.65
Thr	3.63	3.97
Arg	3.66	2.81
Ala	2.92	2.64
Tyr	5.37	4.93
Cys-s	0.08	0.08
Val	6.71	6.37
Met	2.67	2.73
Phe	4.98	4.86
Ile	4.78	5.54
Leu	8.21	8.51
Lys	7.95	7.28
Pro	9.84	13.75
Trp	1.05	0.87
Hydrphopic[c] group	54.58	58.01
Ionzable[D]	43.70	**39.87**

Table 2. Degree of hydrolysis of bovine and buffalo case in hydrolysates.

Time	Trypsin hydrolysis		Alcalase hydrolysis	
	CN	CB	CN	CB
10	19.51± 1.60	18.29±1.50	35.67±1.3	32.93±1.4
20	32.93±1.77	30.49±1.8	48.84±1.22	45.55±1.2
30	45.12±1.5	43.90±1.67	57.62±1.01	54.88±1.03
60	53.66±0.74	51.22±0.55	74.63±0.78	71.34±0.76
90	57.32±0.63	54.88±0.49	84.51±0.45	90.02±0.58
120	60.98±0.67	58.54±0.52	92.26±0.2	86.43±0.3

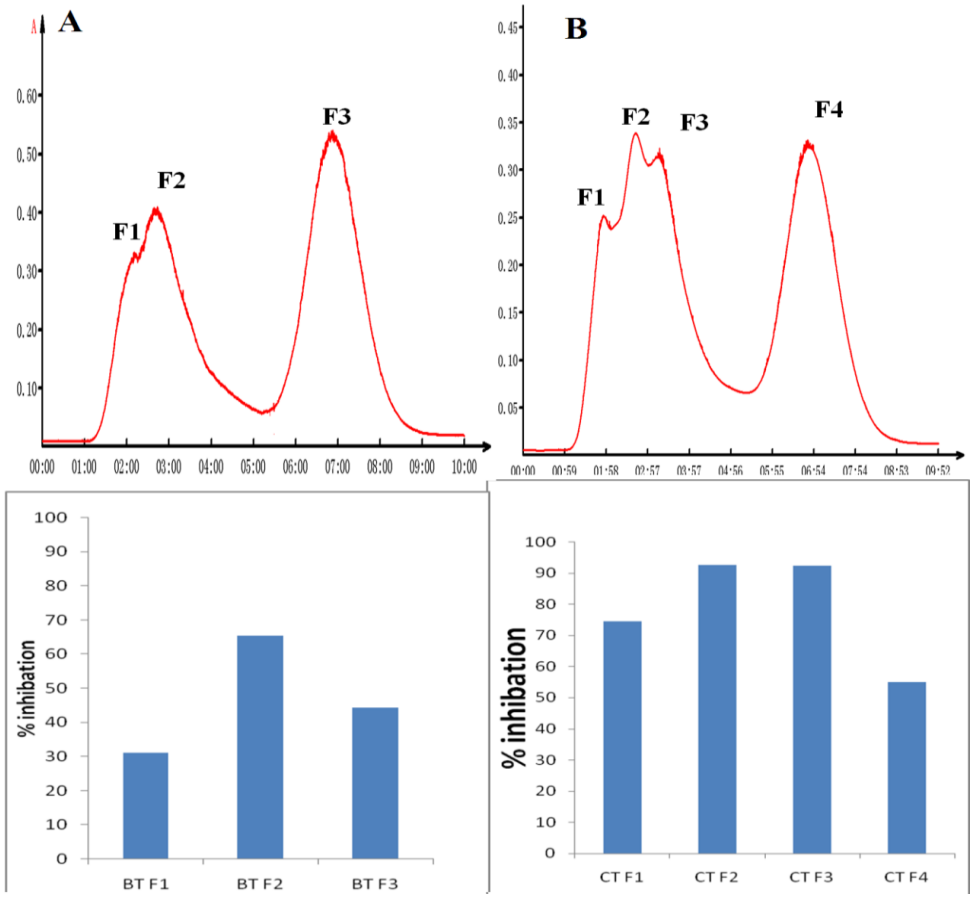

Fig. 1. Antioxidant activity of .Sephadex G-15 gel filtration chromatography Fraction > 1KDaBffalo casein (A) and bovine casein (B) by trypsine hydrolysis.

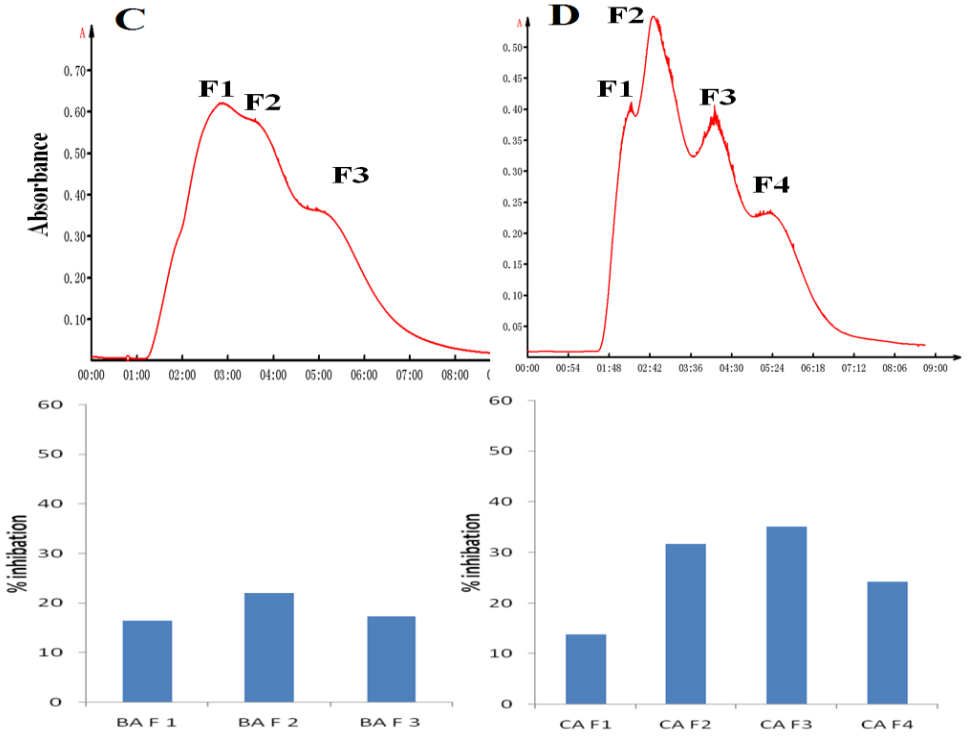

Fig. 2. Antioxidant activity of Sephadex G-15 gel filtration chromatography Fraction > 1 KDaBffalo casein (C) and bovine casein (D) by Alcalase hydrolysis