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The effect of phosphorylation treatment on enzymatic hydrolysis characteristics of casein

Feng Jia, Jinshui Wang*
College of Biological Engineering, Henan University of Technology, Zhengzhou 450001, (P.R.CHINA)
E-mail: jinshuiw@163.com (Wang JS)

ABSTRACT

The caseins were phosphorylated by sodium tripolyphosphate (STP), and then were hydrolyzed by trypsin. The effect of phosphorylation treatment on enzymatic hydrolysis characteristics of casein was investigated with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), high-performance size exclusion chromatography (HPSEC) and ultraviolet absorption. The results showed that phosphorylation treatment improved the degree of hydrolysis of casein. The MW of phosphorlysated casein was decreased at different degrees, and it reached the highest degree for 3h. What's more, compared with the control, the contents of peptide (molecular weight between 20 ku and 35 ku) were significantly increased. However, the ultraviolet absorption spectra of solution did not changed obviously among the phosphorlysated caseins.

KEYWORDS

Casein;
Phosphorylation;
Hydrolysis degrees;
Molecular weight distribution;
SDS-PAGE;
Ultraviolet absorption.

INTRODUCTION

Approximately 80% of the protein of milk is casein[1]. For the isoelectric point of casein is at around pH4.6, it is easy to produce clots that is slowly digested in human's stomach. However, this problem can be solved by modification of casein with chemical reagents, such as phosphorylation, succinylation, glycosylation and acetylation, which have been used to improve the functional properties of caseins. For example, there was an increase in the solubilities of casein and co-precipitate near their isoelectric points upon phosphorylation[2]. Moreover, the enzymatic modification of casein can produce lots of peptides, which have many biological activities such as antimicrobial[3], antioxidant[4], anticariogenic[5], immunomodulation[6], and binding of many important minerals, including calcium and iron[7]. The phosphorylation modification can be attached to the oxygen of seryl, threonyl, aspartyl (β-carboxyl) and tyrosyl residues and via nitrogen to lysyl (ε-amino) and histidyl (1 and 3) residues. Phosphorylation of proteins has been proven as an efficient strategy to improve the functional properties of proteins[8, 9]. Li et al reported that the dry phosphorylation method can improve the digestibility of ovalbumin[10]. The results of studies showed that sodium tripolyphosphate (STP), which was used by the FDA as a kind of food additives[11], may be improved the characteristics of enzymatic hydrolysis

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in some food proteins.

Enzymatic hydrolysis is an important method to enhance the functional properties of proteins commonly used in the modification of protein structure. The significant differences in molecular weight distribution and structure characteristics of casein were found between the casein hydrolysates and the control samples[12]. Proteins by phosphorylation could decrease in amino acid content and increase in larger peptide content (>1450 u), and reduce the bitterness to discernible level, indicating that phosphorylation before hydrolysis could be a potential means to prevent the evolution of bitter flavor which restricts the practical uses of the hydrolysates [13]. However, little information is available for the phosphorylation on enzymatic hydrolysis characteristics of casein.

For this reason, we treated the casein with STP for phosphorylation and with trypsin for hydrolysis. This study was conducted to investigate the hydrolysis degrees (DH), molecular weight distribution (MWD) and ultraviolet absorption (OD) of the casein under phosphorylation.

MATERIALS AND METHODS

Materials

Casein, which contained 82.84 wt% proteins, was obtained from the Dongfanghui chemical company of Henan, and trpsin (1.13×10^5 u/g) was bought from Amersco Company.

Acetonitrile of HPLC grade was purchased from VBS Biologic Inc, and water was doubly distilled. The other chemical reagents were analytical grade or excellent level of pure.

Preparation of phosphorylated casein

Casein was dissolved in the water, which contained 5% (w/v) casein, and then added to STP at a final concentration of 5%. The solution was prepared at pH 9.0 with 1 mol NaOH under constant stirring, and incubated at 30°C in rotatory shakers. Then the levels of phosphorylation in different time were determined.

Tripsin hydrolysis of casein and phosphorlated casein

After phosphorylation of casein, 50 mg/mL of casein was digested with tripsin (1.25 mg/mL) at 40°C and pH8.0 in a batch stirred tank reactor. Each aliquot was heated in boiling water for 10 min to inactivate the tripsin activities and stop the reaction. The samples were lyophilized, and then stored at -20°C. The percentage of peptide bond cleaved during the enzymatic reaction, the hydrolysis degrees, was measured using the pH-stat method, as described by Su[14].

Determination of the degree of phosphorylation

Five mL of the solution after phosphorylation of casein were extracted to add the same volume with 10% trichloroacetic acid solution. The denaturated protein were precipitated at 5 000 g for 10 min, and the supernatant was extracted to adjust the pH 3.8 to 3.9 with 1 moL zinc acetate. The ingredients of precipitation are $Zn_2P_20_7$, which could dissolve in the ammonia buffer solution at pH 10, then added 5 drops of chrome black T as an indicator with 0.02 mol/L EDTA disodium standard solution titration. The color of the solution at the end-point changed from purple to blue.

The degree of phosphorylation (%) = $C \times (V_2 - V_1) \times Mp / 2 M$ (unit: mg P/g Pr)

(Note: C, concentration of EDTA disodium standard solution, mol/L; V_1, volume of EDTA disodium titration standard solution titrated by blank, mL; V_2, volume of EDTA disodium standard solution titrated by samples, mL; Mp, the relative atomic mass of phosphorus, 30.97, mol/kg; M, the molecular mass of casein, g.)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was performed using a vertical gel electrophoresis system with a 0.75 mm thickness. Samples and a protein molecular weight ladder were dispersed in1.0 mol Tris-HCl buffer (pH 6.8) containing 4% (w/v) SDS, 10% (v/v) β - mercaptoethanol, and 0.01% (v/v) bromophenol blue and heated at 100°C for 10 min. The resolving gel contained 15% (w/v) acrylamide (2.6% Bis) and 0.1% (w/v) SDS in 1.5 mol Tris–HCl buffer (pH 8.9) and the stacking gel contained 4% (w/v)

acrylamide and 0.1% (w/v) SDS in 1.0 mol Tris–HCl buffer (pH 6.8). The gels were run at constant current of 30 mA per gel at 10°C for approximately 2 h. Proteins in the gels were then destained using 0.1% silver staining protocol (Swain & Ross). The gels were scanned using a Digital Single Lens Reflex camera. The data was analyzed with the software Quantity One.

The size exclusion chromatography analysis

The high-performance size exclusion chromatographic (HPSEC) system was Agilent LC1260 (Agilent Technologies, Waldbronn, Germany), and the column was PL aquagel-OH MIXED-H (8 um, 300 mm×7.5 mm I.D.). The column was thermostated at 30°C and eluted with a mixture of water–ACN (90:10, v/v). The flow rate was 0.5 mL/min. The elution profiles were monitored by an ultraviolet (UV) detector, and the detection wavelength was 214 nm.

The ultraviolet absorption analysis

The lyophilized samples were dissolved in ultrapure water to obtain a working solution of 0.5 mg/mL. The wavelength ranges of ultraviolet scanning were from 220 nm to 300 nm. The wavelength of sampling interval was 1 nm. The samples were detected using the Epoch Multi-Volume Spectrophotometer System (Biotek, USA).

RESULTS AND DISCUSSION

Effect of phosphorylation treatment on casein

The results of the degrees of phosphorylated was shown in Figure 1. As seen in Figure 1, the degrees of phosphorylation were different for 0.5, 1, 2, 3, 4 and 5 hr with 5% STP. The phosphorylation was rapid within the 3 hour of reaction, and the degree of phosphorylation was 0.018 mg/mg. The phosphorylation of soybean protein isolated was treated with $POCl_3$, and phosphorus content was 0.014 mg/mg in isolated soybean protein [15]. However, the degrees of phosphorylation in casein declined with the reactions proceeding.

Effect of the hydrolysis degree of casein and phosphorylated casein

Aliquots of casein hydrolysis were removed to determine the DH at 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 80 min, 100 min, 120 min and 150 min, respectively Figure 2. Figure 2 showed that the change trend of DH of the three sample increased significantly ($p<0.01$) in 1h. Within the first 3 hours, the DH did not a significant increase. The results of DH of phosphorylated casein from 10 min to 72 hr were showed in Figure 2 and Figure 3. As can be seen from the Figure2 and Figure 3, the DH of phosphorylated casein for 30 min or 3 hr were similar with the control. While the DH of phosphorylated casein for 3hr significantly increased than both the control group and phosphorylated casein for 2hr. From 12 hr to 72 hr, the significant differences of the DH of phosphorylated casein were found in significantly increase at treated for 3 hr. The DH of phosphorylated casein for 2 hr did not significantly increase compared with control.

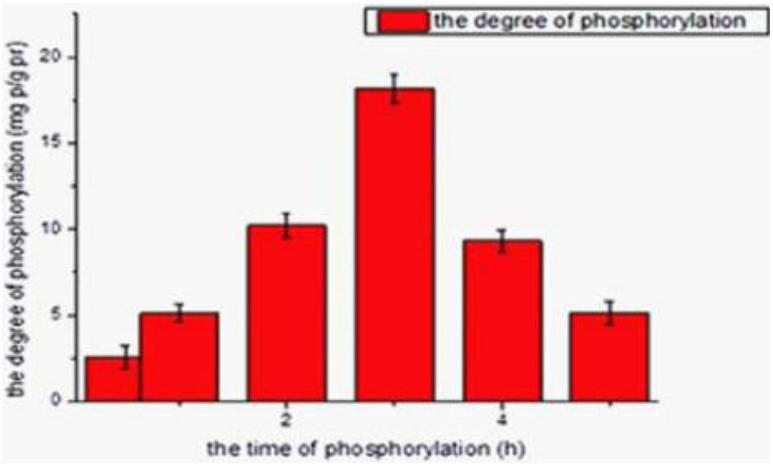

Figure 1 : The degrees of phosphorylated casein

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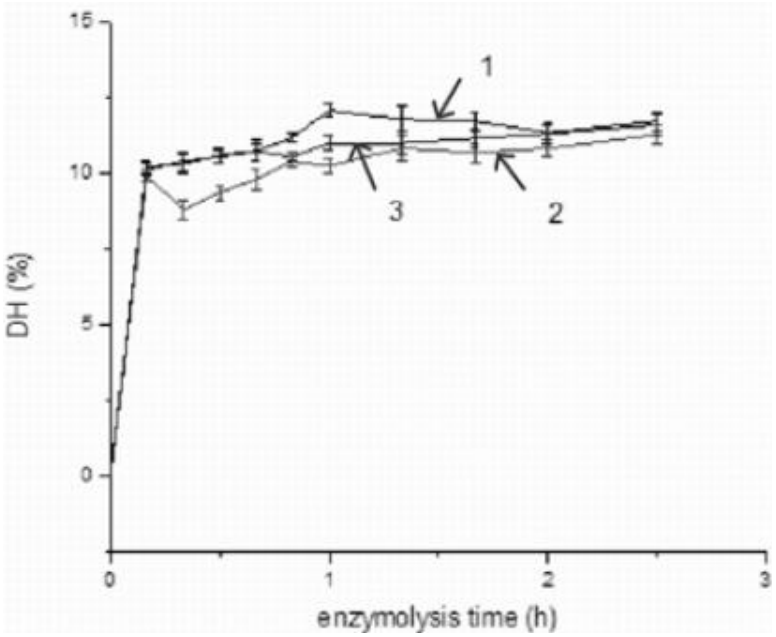


Figure 2 : The hydrolysis degrees of (1) casein, (2) phosphorylated casein for 2h, and (3) phosphorylated casein for 3h, respectively

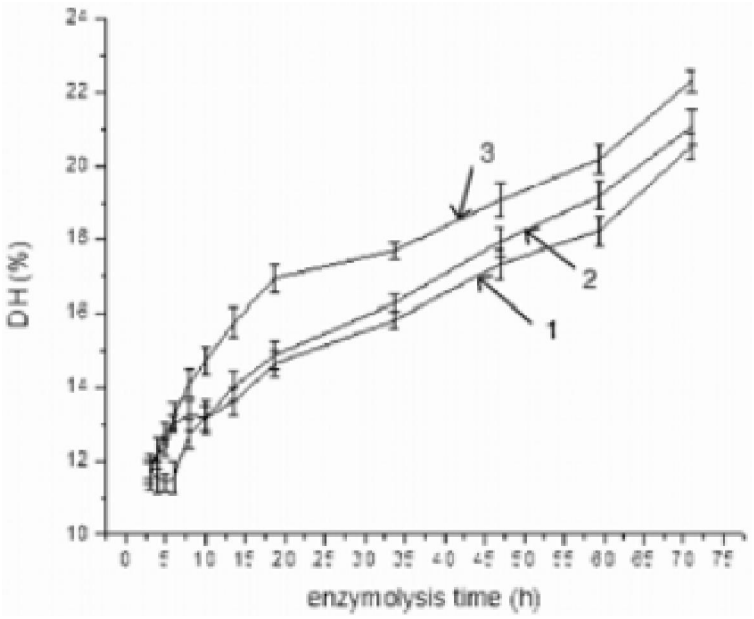
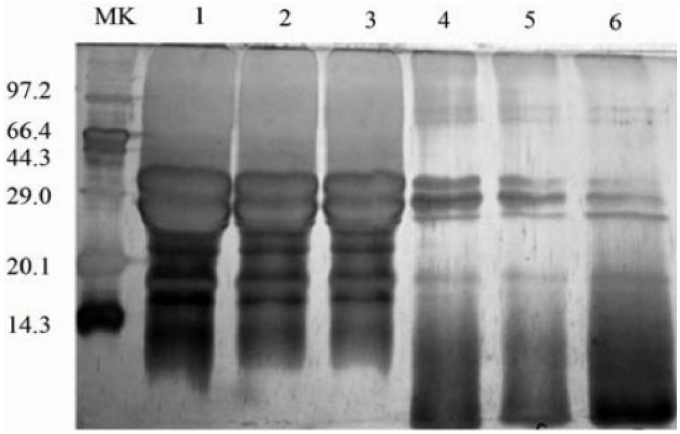


Figure 3 : The hydrolysis degrees of (1) casein, (2) phosphorylated casein for 2 h and (3) phosphorylated casein for 3 h, respectively

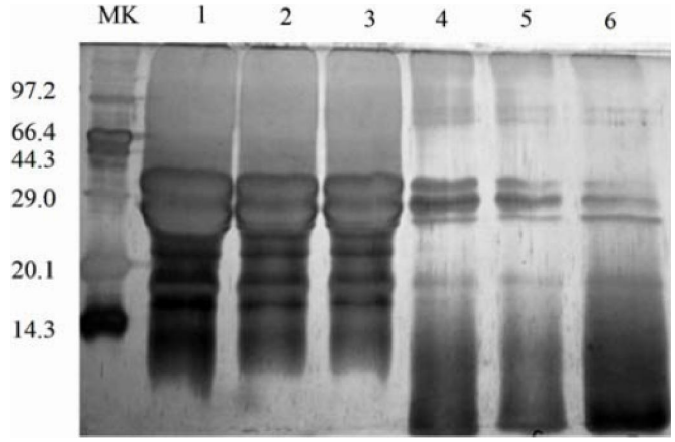
Caseins, which were a dynamic balance form of polymers and monomers in solution, were hydrolyzed with trypsin to form the number of monomers and the polymers. And the size and molecular weight of polymers would decreace, because the repulsive force between monomers might be reduced[16]. The introduction of the phosphate could increase the repulsive force between the molecules of caseins, and improve the amount of the monomers, and enhance the efficiency of enzymatic hydrolysis. The results showed that phosphorylation modification could improve DH of samples at different levels. The higher degree of phosphorylated casein, the higher DH of casein.

The analysis of the SDS-PAGE of casein, phosphorylated casein and its hydrolysates

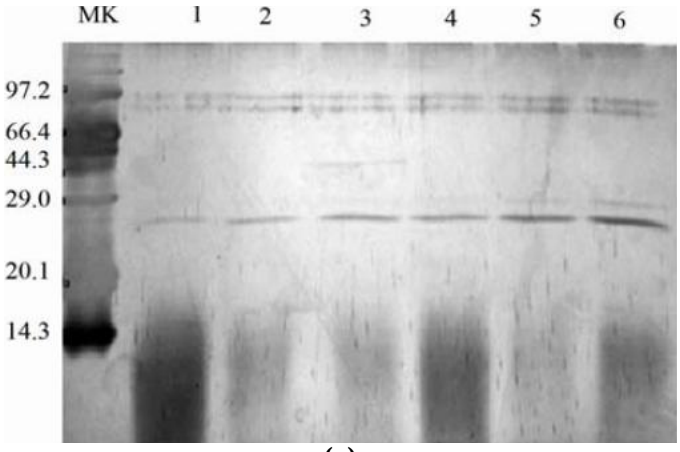
As shown in Figure 4, the changes of molecular



(a)



(b)



(c)

a, MK: molecular mass standards; Lane 1: casein; Lane 2: casein phosphorylated for 2 hr; Lane 3: casein phosphorylated for 3 hr; Lane 4: hydrolysate of casein hydrolyzed for 10 min; Lane 5: hydrolysate of casein phosphorylated for 2 hr hydrolyzed for 10 min; Lane 6: hydrolysate of casein phosphorylaed for 3 hr hydrolyzed for 10 min; b, MK: molecular mass standards; Lane 1: hydrolysate of casein hydrolyzed for 30 min; Lane 2: hydrolysate of casein phosphorylated for 2 hr hydrolyzed for 30 min; Lane 3: hydrolysate of casein phosphorylaed for 3 hr hydrolyzed for 30 min. Lane 4: hydrolysate of casein hydrolyzed for 1 hr; Lane 5: hydrolysate of casein phosphorylated for 2 hr hydrolyzed for 1 hr; Lane 6: hydrolysate of casein phosphorylaed for 3 hr hydrolyzed for 1 hr; c, MK: molecular mass standards; Lane 1: hydrolysate of casein hydrolyzed for 2hr; Lane 2: hydrolysate of casein phosphorylated for 2 hr hydrolyzed for 2 hr; Lane 3: hydrolysate of casein phosphorylaed for 3 hr hydrolyzed for 2 hr. Lane 4: hydrolysate of casein hydrolyzed for 4 hr; Lane 5: hydrolysate of casein phosphorylated for 2 hr hydrolyzed for 4 hr; Lane 6: hydrolysate of casein phosphorylaed for 3 hr hydrolyzed for 4 hr.

Figure 4 : SDS-PAGE stained by silver of casein and phosphorylated casein hydrolyzed by trpsin

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TABLE 1 : The molecular weight distribution of Figure 4a

Band Number	The molecular weight（ku）					
	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
1	37.4	43.5	76.8	35.0	33.6	30.3
2	29.8	35.7	42.0	33.2	30.1	27.8
3	23.8	25.5	35.2	30.0	20.5	20.8
4	21.5	23.8	25.5	20.3	10.0	16.6
5	16.6	21.0	23.8	9.1	6.3	13.9
6	11.8	16.4	16.8	5.7	5.6	12.4

TABLE 2 : The molecular weight distribution of Figure 4b

Band Number	The molecular weight（ku）					
	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
1	29.0	29.8	29.2	29.2	29.2	28.8
2	16.2	12.5	4.5	12.3	4.6	4.8

TABLE 3 : The molecular weight distribution of Figure 4c

Band Number	The molecular weight（ku）					
	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
1	27.3	27.5	27.8	28.0	27.8	27.5
2	14.1	-	-	4.3	-	-

weight of casein, phosphorylated casein and its hydrolysates were investigated using SDS-PAGE of proteins. The data of SDS-PAGE in TABLE 1, TABLE 2, and Table 3 were obtained by analysing the electrophoretograms with Qualtity One. The molecular weight distribution of Figure 4a described in TABLE 1. As can beseen from TABLE 1, it is clear that the MW of phosphorlysated casein increased at different degrees, and the MW of casein phosphorylated for 3 hr was the largest compared with the others. Moreover, the casein of the lane 5 has six kinds of molecular weights. The changes of casein and phosphorylated casein's composition could be seen from hydrolyzed for 10 min Figure 4a. The MW of hydrolysates would degrade, and the DH of casein phosphorylated for 2 hr would be higher than that for 3 hr.

The numbers of the lane in Figure 4b and Figure 4c were less than Figure 4a. The MW of casein had taken place obvious changes after hydrolyzed for 30 min, because the low molecular peptides in the hydrolysates were detected. The MW of peptide was appproximate 29 ku in the hydrolysates for 1 hr TABLE 2. While hydrolysated for 4 hr, the MW of about 80% peptide were less than 4 ku in enzymolysis of phosphorylated casein, and the lanes of peptides were fuzzy in electrophoresis figure (Figure 4 c).

HPLC analysis of hydrolysates of casein

As shown in TABLE 4, the retention time of phosphorylated casein 4 and 7 were delayed than casein 1, and the peaks of phosphorylated casein migrated to the left in electrophoretogram, which suggested that there were more low molecular peptides in solution. And the molecular weight distribution of less than 20 ku in phosphorylated casein 4 and 7 had increased from 4.35% to 16.81% and 12.73%, respectively (TABLE 4). Within the first 12 hr, the rate of the molecular weight (less than 20 ku) of peptides in the enzymolysis liquid of phosphorylated casein were 56.09%, and 56.26%, respectively, while the control were 65.72%. In addition, the peptide contents of the molecular weight (between 20 ku and 35 ku) were significantly increased. The numbers of peptides the molecular weight (larger than 40 ku) sharply reduced with extending the time for enzymatic hydrolysis, and the molecular weight of peptides (less than 20 ku) in the enzymatic hydrolysate were rapidly increased within the first hour. As shown in Fig-

TABLE 4 : The molecular weight distribution of phosphorylated casein

Treatments	Molecular weight distribution (%)				
	<20 ku	20-30 ku	30-35 ku	35-40 ku	>40 ku
1	4.35	1.23	0.84	1.09	88.5
2	55.51	10.12	4.62	4.91	24.85
3	65.72	10.54	4.07	4.33	16.22
4	16.81	14.1	5.31	3.39	61.32
5	56.09	23	6.32	4.39	16.6
6	64.58	14.6	5.12	3.22	12.48
7	12.73	12.1	4.77	4.57	66.97
8	56.26	16.67	6.05	4	17.04
9	63.55	14.87	5.34	3.24	12.99

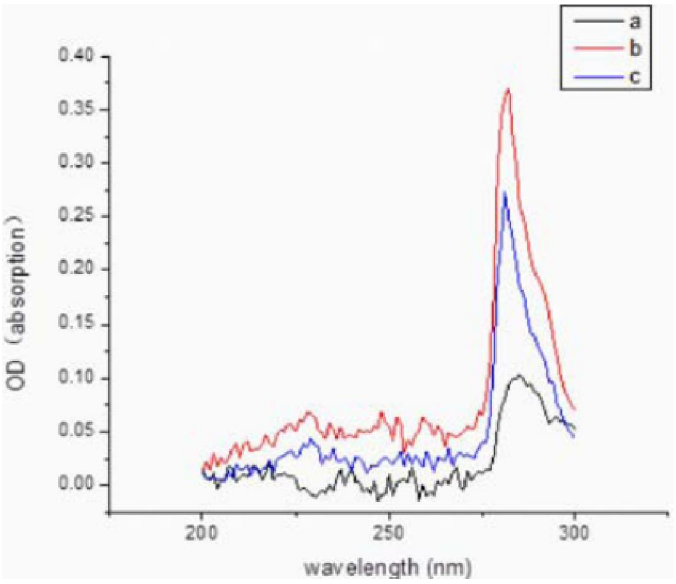

Figure 5 : The UV scanning curves of a (casein), b (casein phosphorylated for 3 hr) and c (casein phosphorylated for 2 hr)

ure 5, phosphorylation treatment was useful to produce the new peptides, which one more peak in the sample 5, 6, 8 and 9 than sample 2 and 3, respectively.

Caseins in the solutions was envisioned as a dynamic system of casein aggregates and monomers [14]. The proteins of casein were gradually hydrolyzed into small molecular peptides in the enzyme reaction, and pancreatin attacked different caseins with varying hydrolysis rates (β-casein >αs1-casein > αs2-casein > κ-casein) [17]. Compared with the control group, phosphorylated casein could change the dynamic system, and produce more peptides of molecular weight between 20 ku and 35 ku. The resluts showed that phosphorylation contributed to form the low molecular peptide in hydrolyzed solution. The new peptides can be observed at the retention time from 1 min to 20 min, and these results were similar to those obtained by Toldrá et al. [18].

Effect of the UV scanning curve of enzymatic hydrolysates

Different degree of phosphorylation had different absorption at the maximum absorption wavelength, and absorbance was positively correlated with phosphorylation levels Figure 5. Ultraviolet spectrophotometer was commonly used to detect the concentration of the protein and others. A research described a novel method to measure relative and absolute amounts of non-glycosylated, de-glycosylated, and total glycosylated protein using an HPLC–UV–MS

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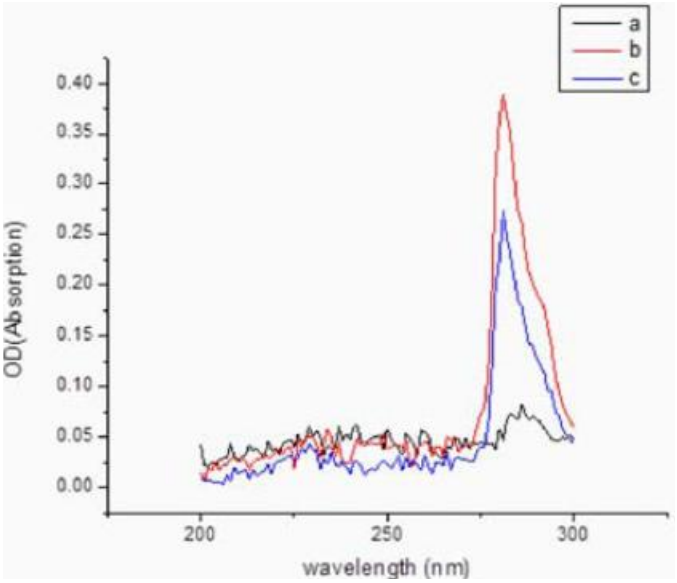

Figure 6 : The UV scanning curves of the hydrolysate for 10 min.; a, the hydrolysate of casein; b, the hydrolysate of casein phosphorylated for 3h; c, the hydrolysate of casein phosphorylated for 2 hr.

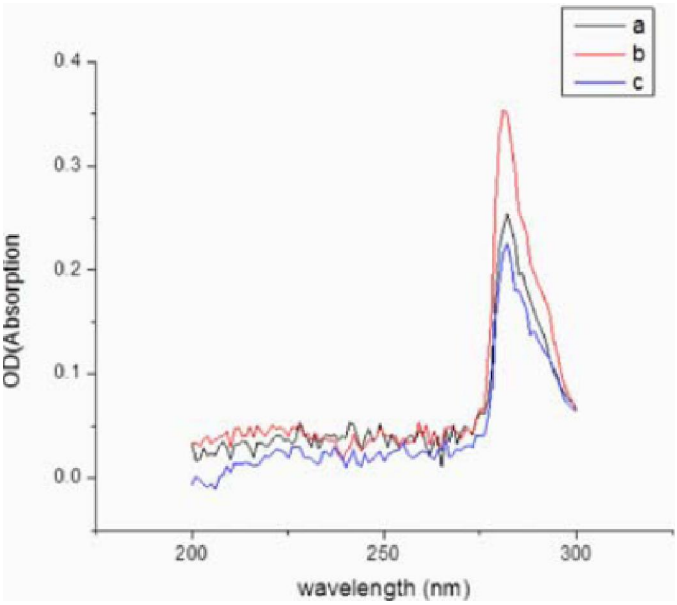

Figure 7 : The UV scanning curves of the hydrolysate for 72 hr.; a, the hydrolysate of casein; b, the hydrolysate of casein phosphorylated for 3 hr; c, the hydrolysate of casein phosphorylated for 2 hr.

methodology accurately [19].

As shown in Figure 6 and Figure 7, the maximum absorption wavelength of enzymatic hydrolysates shifted to the left at 280 nm, which there were some new substances produced as hydrolysis processing, and the OD of the control increased from 0.104 to 0.2235 as hydrolysis for 72 h as well. It means that the number of peptides that contained Trp, Phe and Tyr were increased. The maximum absorption wavelength shifted to the left at 280 nm.

CONCLUSIONS

The results showed that phosphorylation treatment improved the degree of hydrolysis of casein; The MW of phosphorlysated casein was decreased at different degrees, and it reached the highest degree for 3h. What's more, compared with the control, the contents of peptide (molecular weight between 20 ku and 35 ku) were significantly increased. However, the ultraviolet absorption spectra of solution did not changed obviously among the phosphorlysated

caseins.

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REFERENCES

[1] T.L.Mcmeekin, B.D.Polis; Milk Proteins. Advances in Protein Chemistry, 5201-228 **(1949)**.

[2] S.K.Nayak, Arora Sumit, J.S.Sindhu, R.B.Sangwan; Effect of chemical phosphorylation on solubility of buffalo milk proteins, International Dairy Journal, **16(3)**, 268-273 **(2006)**.

[3] M.S.Arruda, F.O.Silva, A.S.Egito, T.M.S.Silva, J.L.Lima-Filho, A.L.F.Porto, K.A.Moreira; New peptides obtained by hydrolysis of caseins from bovine milk by protease extracted from the latex Jacaratia corumbensis, LWT - Food Science and Technology, **49(1)**, 73-79 **(2012)**.

[4] D.D.Kitts; Antioxidant properties of casein-phosphopeptides, Trends in Food Science & Technology, **16(12)**, 549-554 **(2005)**.

[5] A.T.Andrews, R.J.H.Williams, V.L.Brownsell, F.H.Isgrove, K.Jenkins, A.D.Kanekanian; β-CN-5P and β-CN-4P components of bovine milk proteose–peptone: Large scale preparation and influence on the growth of cariogenic microorganisms, Food chemistry, **96(2)**, 234-241 **(2006)**.

[6] R.Pizzano, M.A.Nicolai, P.Padovano, P.Ferranti, F.Barone, F.Addeo; Immunochemical evaluation of bovine beta-casein and its 1-28 phosphopeptide in cheese during ripening Journal of Agricultural and Food Chemistry, 484555-4560 **(2000)**.

[7] Meisel Hans, Olieman Cornelis; Estimation of calcium-binding constants of casein phosphopeptides by capillary zone electrophoresis, Analytica chimica.acta., **372(1–2)**, 291-297 **(1998)**.

[8] T.Aoki, K.Kitahara, T.Fukumoto, Y.Sugimoto, H.Ibrahim, T.Kimura, Kato Yasuko; Matsuda Tsukasa Improvement of functional properties of β-lactoglobulin by conjugation with glucose-6-phosphate through the Maillard reaction, Food Research International, 30401e406 **(1997)**.

[9] G.Matheis, M.H.Penner, R.E.Feeney, J.R.Whitaker; Phosphorylation of casein and lysozyme by phosphorus oxychloride, Journal of A gricultural and Food Chemistry, **31(2)**, 379-387 **(1983)**.

[10] C.P.Li, H.R.Ibrahim, Y.Sugimoto, H.Hatta, T.Aoki; Improvement of functional properties of egg white protein through phosphorylation by dry-heating in the presence of pyrophosphate, J.Agric.Food.Chem., **52(18)**, 5752-5758 **(2004)**.

[11] Dua Saroj, Mahajan Anupama, Mahajan Amita; Improvement of Functional Properties of Rapeseed (Brassica campestris Var. Toria) Preparations by Chemical Modification, Journal of Agricultural and Food Chemistry, **44(3)**, 706-710 **(1996)**.

[12] Wang Jinshui, Su Yinjie, Jia Feng, Jin Huali; Characterization of casein hydrolysates derived from enzymatic hydrolysis, Chemistry Central Journal, **7(1)**, 1-8 **(2013)**.

[13] Liu Tong-Xun, Zhao Mou-Ming; Thermal pretreatment and chemical modifications as a means to alter hydrolytic characteristics and prevent bitterness in hydrolysates of fishery by-catch (Decapterus maruadsi) protein, International Journal of Food Science and Technology, **45(9)**, 1852-1861 **(2010)**.

[14] Su Rongxin, Qi Wei, He Zhimin, Yuan Suxia, Zhang Yubin; Pancreatic hydrolysis of bovine casein: Identification and release kinetics of phosphopeptides, Food chemistry, **104(1)**, 276-286 **(2007)**.

[15] Hirotsuka Motohiko, Taniguchi Hitoshi, Narita Hiroshi, Kito Makoto; Functionality and digestibility of a highly phosphorylated soybean protein, Agricultural and Biological Chemistry, **48(1)**, 93-100 **(1984)**.

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[16] Su Rongxin, He Zhimin, Qi Wei; Pancreatic hydrolysis of bovine casein: Changes in the aggregate size and molecular weight distribution, Food Chemistry, 107151-157 **(2008)**.

[17] Su Rongxin, Liang Miao, Qi Wei, Liu Rui, Yuan Suxia, He Zhimin; Pancreatic hydrolysis of bovine casein: Peptide release and time-dependent reaction behavior, Food Chemistry, **133(3)**, 851-858 **(2012)**.

[18] Toldrá Fidel, Molina Laura, Vinuesa Sergio, Aristoy M-Concepción; Peptide generation from casein hydrolysis by immobilised porcine cathepsins, Food Chemistry, 92227-233 **(2005)**.

[19] Karnoup S.Anton, Kuppannan Krishna, Young A.Scott; A novel HPLC-UV-MS method for quantitative analysis of protein glycosylation, Journal of chromatography B, **858(2)**, 178-191 **(2007)**.