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Short Communication

STANDARDIZATION OF GOAT MILK WHEY PROTEIN ENZYMATIC HYDROLYSIS CONDITIONS WITH PAPAIN

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SUMMARY

Goat milk is highly nutritious and is consumed in many countries, but the development of functional foods from goat milk has been slow compared to that for other types of milk. The aim of this study was to develop a goat milk whey protein hydrolysate (GMWPH) with enhanced antioxidant property and better Ca^{2+} chelating activity. Goat milk whey protein was hydrolyzed with commercial food-grade Papain enzyme under various conditions of incubation temperature (30 to 70 ºC), incubation time (30 to 300 min), enzyme concentration level (0.25 to 2%) and pH (6 to 10) of the enzyme reaction to achieve the best hydrolysis. The hydrolysates were analyzed for degree of hydrolysis (DH), antioxidant activity (ATBS) and calcium chelating capacity. It was found that treatment with papain at 50 ºC incubation temperature, 180 min incubation time, 1.0% enzyme concentration and 7.0 pH effectively hydrolyzed the goat milk whey proteins, as determined by SDS-PAGE and measurement of non protein nitrogen content. Hydrolysis with Papain resulted in a significant increase in antioxidant and Ca^{2+} chelation property. Hence, the GMWPH may be useful for development of novel foods for infants, and the elderly osteoporosis patients.

Keywords: Goat whey protein, papain hydrolysis, degree of hydrolysis, antioxidant, calcium chelating activity

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Goat milk chemical structure is amazingly similar to mother's milk (Rafter, 2003). It's whey protein peptides are rich in amino acids which are highly digestible and have positive effect on satiety and mood, improve morning alertness and brain-sustained attention processes (Markus, 2005).

The identification and development of whey protein-derived peptides with antioxidant properties has also attracted increased attention due to heightened safety concerns over the use of synthetic antioxidants such as BHT to inhibit lipid oxidation and improve shelf-life meat products. In the light of above discussion, to prepare goat milk whey protein bioactive peptides and their potential applications for calcium encapsulation to develop calcium enriched functional foods, the present study was designed to preparation and characterization of bioactive peptides from goat milk whey proteins.

Preparation of goat milk whey proteins

Fresh whole pooled goat milk sample was aseptically collected in triplicate in sterilized sample containers from the Goat Yard, National Dairy Research Institute, Karnal, Haryana. The milk was defatted in a refrigerated centrifuge at 4,000 rpm for 30 min. at 4 °C to separate cream from milk. The pH of defatted milk was adjusted to 4.6 with 1 N HCl at temperature 20 ºC with slow stirring and was held at room temperature for 30 min for clear separation of casein and whey. The whey was separated from the precipitated case in curd by centrifugation at 8000 rpm/min, at 4 ºC for 20 min and filtering through four layered muslin cloth. The samples were pre-frozen at -18 ºC for 24 h and then placed in a freeze-dryer (SCIENTZ-10N, at NDRI, Karnal) and vacuum freeze-dried at 50 ºC and 2–10 Pa to obtain freeze-dried powder samples, and stored at -20 ºC until further use.

Enzymatic hydrolysis of goat milk whey proteins

Food-grade commercial protease (Papain) was purchased from Sigma–Aldrich Chemical Co. USA. Incubation temperature (30-70 °C), incubation time (30-300 min), enzyme concentration (0.25-2.0%, w/w) and pH (6 to 10) were varied to determine the optimal conditions for hydrolysis. Upon completion of hydrolysis reactions, the samples were heated at 90 °C for 15 min to inactivate the enzymatic activity. They were freeze-dried and analyzed by SDS-PAGE. The degree of hydrolysis in each condition was then determined by quantification of nonprotein nitrogen (NPN).

The degree of hydrolysis (DH) of whey hydrolysates was determined by the percentage of solubilized protein in 10% (w/v) trichloroacetic acid (TCA), according to Hoyle and Merritt (1994), with modifications. The DH was calculated according to the following equation:

$$DH\,\% = (h / h_{tot}) \times 100$$

Where, $h = (\text{serine-NH2} - \beta) / \alpha$ meqv / g / protein.

α, β and h_{tot} constants for whey protein are 1.039, 0.383 and 8.2, respectively.

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SDS-PAGE

This procedure was carried out on a 12.5% acrylamide gel, as described by Laemmli (1970). Electrophoresis was performed at 20 mA for 1 h, using a Mini-Protean® Tetra System and PowerPacTM HV (Bio-Rad, Hercules, USA). The gel was stained for 1 h with a Coomassie blue solution and analysis of the bands on the gel was performed using a Molecular Imager® GelDocTM XR plus Imaging system and the Image LabTM software version 5.1 (Bio-Rad).

Determination of NPN

NPN contents were measured by the Folin-Lowry method (Lowry *et al.*, 1951). In brief, 2 mL of the hydrolyzed sample and the same volume of 24% trichloroacetic acid solution were mixed, incubated for 30 min, and centrifuged at 3,000 rpm for 20 min (Labogene 1736R). Next, 1 mL of the supernatant was transferred to a fresh test tube, 5 mL of the assay reagent was added, the mixture was incubated for 15 min at room temperature, and then mixed with 0.5 mL of the phenol reagent. After 30 min of incubation, the absorbance of the mixture was measured at 750 nm. The standard solution was prepared from bovine serum albumin.

ABTS+ radical-scavenging activity

The spectrophotometric analysis of ABTS+ radical-scavenging activity was determined according to method described by described by Salani *et al.* (2011). The ABTS+ activity was calculated by using the following formula:

$$\text{ABTS activity (\% inhibition)} = \frac{0.7\text{-At20} \times 100}{0.7}$$

Calcium Chelating Activity

Calcium-binding capacity was defined as the content of calcium (µg) bound with peptide (mg) after the chelation reaction. It was measured with ortho-cresolphthale incomplex one reagent using complexometric titration method as followed by Xixi *et al.* (2015) with some modifications. 250ml of 2.5% (w/v) calcium-chelating peptide and 75 ml of 1% (w/v) $CaCl_2$ solutions were prepared in deionized water.The absorbance at 570 nm was determined after adding the working solution to the sample.

Statistical analysis

The results were presented as mean+SE, and differences were analyzed using the SAS/PROC GLM software (SAS version 9.1; SAS Institute Inc., USA). Statistical significance was assumed at $p<0.05$.

Standardization of incubation temperature

The hydrolysates of GMWP using papain enzymes were compared with the standard molecular weight marker using SDS-PAGE analysis (Fig. 1A).

SDS-PASE showed that the hydrolyzation of GMWP with papain enzyme was increased as the incubation temperature increased significantly till 50 ºC. These findings were further confirmed with NPN method (Fig. 1B). Fig. 1A and 1B revealed that the hydrolyzation of GMWP was optimum at 50 ºC with papain enzyme. Wang *et al.* (2020) also followed 50 ºC as optimum temperature for hydrolyzation of camel and cow whey protein with papain.

Optimization of incubation time

The hydrolysates of GMWP using papain enzyme were done at different incubation times from 30 to 300 min, and hydrolysis were compared with the standard molecular weight marker using SDS-PAGE analysis (Fig. 2A).

SDS-PASE showed that the hydrolyzation of GMWP with papain enzyme was increased as the incubation time increased up to 180 min. These findings were further confirmed with NPN method (Fig. 2B). Fig. 2A and 2B revealed that the hydrolyzation of GMWP did not increase significantly (<0.05) after 180 min of incubation time. Similar results were also reported by Liu *et al.* (2012).

Optimization of incubation enzyme concentration

Different concentrations of papain enzyme were used for hydrolysis of GMWP and hydrolysates were compared with the standard molecular weight marker using SDS-PAGE analysis (Fig. 3A).

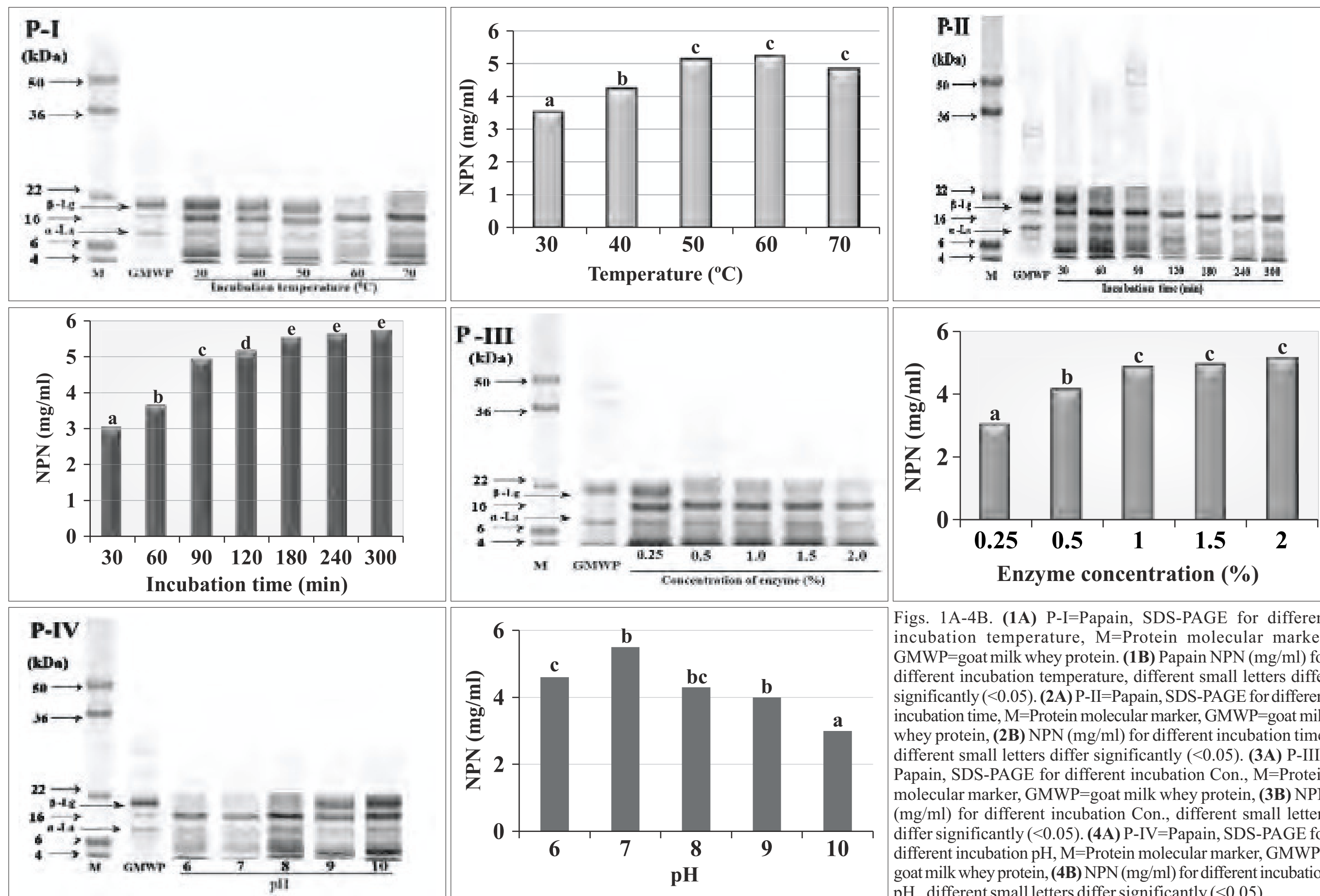
SDS-PASE showed that the hydrolyzation of GMWP with papain enzyme was increased as the enzyme concentration increased up to 1.0 % level. These findings were further confirmed with NPN method (Fig. 3B). Fig 3A and 3B revealed that the hydrolyzation of GMWP did not increase significantly (<0.05) after 1.0% level of enzyme concentration. These results are in accordance with the line of Liu *et al.* (2012).

Optimization of incubation pH

The hydrolysis of GMWP using papain enzymes were done at different incubation pH levels and hydrolysates were compared with the standard molecular weight marker using SDS-PAGE analysis (Fig. 4A).

SDS-PASE showed that the hydrolyzation of GMWP powder with papain enzyme was highest at pH 7.0 level. These findings were further confirmed with NPN method (Fig. 4B).

Fig. 4A and 4B revealed that the hydrolyzation of GMWP was optimum at 7.0 pH level with papain enzyme. Lafarga *et al.* (2016) reported that papain is most active in



Figs. 1A-4B. **(1A)** P-I=Papain, SDS-PAGE for different incubation temperature, M=Protein molecular marker, GMWP=goat milk whey protein. **(1B)** Papain NPN (mg/ml) for different incubation temperature, different small letters differ significantly (<0.05). **(2A)** P-II=Papain, SDS-PAGE for different incubation time, M=Protein molecular marker, GMWP=goat milk whey protein, **(2B)** NPN (mg/ml) for different incubation time, different small letters differ significantly (<0.05). **(3A)** P-III= Papain, SDS-PAGE for different incubation Con., M=Protein molecular marker, GMWP=goat milk whey protein, **(3B)** NPN (mg/ml) for different incubation Con., different small letters differ significantly (<0.05). **(4A)** P-IV=Papain, SDS-PAGE for different incubation pH, M=Protein molecular marker, GMWP= goat milk whey protein, **(4B)** NPN (mg/ml) for different incubation pH., different small letters differ significantly (<0.05).

Table 1

The percent DH, ABTS and calcium chelating activity of GMWPH with papain

Proteolysis time (Hrs)	Hydrolyzing enzymes Papain		
	DH (%)	ABTS (%)	Ca^+ (%)
1	$8.5^{a} \pm 0.08$	$49.75^{aB} \pm 1.01$	$12.9^{a} \pm 0.11$
2	$9.6^{b} \pm 0.13$	$51.29^{aB} \pm 0.82$	$21.4^{b} \pm 0.09$
3	$12.2^{c} \pm 0.12$	$56.84^{bB} \pm 0.60$	$24.8^{c} \pm 0.13$
4	$14.84^{dc} \pm 0.09$	$57.02^{bB} \pm 0.91$	$28.5^{dc} \pm 0.12$
5	$16.22^{de} \pm 0.14$	$56.43^{bA} \pm 0.74$	$28.9.3^{de} \pm 0.08$
6	$16.7^{e} \pm 0.13$	$57.01^{bA} \pm 0.69$	$29.3^{e} \pm 0.12$

Mean±SE with different small letters superscripts column wise differ significantly ($p \leq 0.05$)

the pH range between 7 and 7.5.

DH, ABTS and Ca^+ chelating activity

The DH is a measure of the extent of hydrolytic degradation of a protein and is the most widely used indicator for comparing different proteolytic processes. Degree of hydrolysis, ABTSand calcium chelating activity of goat milk whey protein hydrolysates by papain was expressed in terms of percentage (%) of hydrolysis carried out under different standardized control conditions (Table 1).

DH for papain enzyme increased with increase in time of hydrolysis from 1 hr to 6 hrs. It was observed that papain enzyme till 4 hrs (14.84%) produced peptides with increased degree of hydrolysis significantly, but after that there was no significant increase in DH. Kumar *et al.* (2016) also reported that the rate of DH increased linearly up to 2 h; thereafter, the rate of DH decreased and, subsequently, it got stabilized in camel milk casein hydrolyses with different enzymes. The reduction in hydrolysis rate over time may indicate the decreased availability of cleavable peptide bonds within the substrate.

The ABTS radical-scavenging activity increased significantly (P<0.05) with the advancement of hydrolysis time up to 4 hrs for papain GMWPH. Similar reports were documented by Kumar *et al.* (2016).

These results indicated that the degree of hydrolyzation by papain enzyme treatment influences the Ca-chelating activity within 4 hrs of the obtained GMWPH and after 4 hrs, there was no significant further increase in Ca-chelating ability, which meant that DH played an important role in the chelating reaction between

GMWPH and Ca ions. Xixi *et. al.* (2015) also indicate that the degree of enzyme treatment influences the Ca-chelating activity of the obtained WPH. Their results also showed that goat milk hydrolysates have higher calcium binding activity than human and sheep milk.

CONCLUSION

The aim of this study was to develop a goat milk protein hydrolysate (GMWPH) with enhanced antioxidant property and better Ca^+ chelating activity. Goat milk whey protein was hydrolyzed with commercial food-grade Papain enzyme under various conditions of incubation temperature, incubation time, enzyme concentration level and pH of the enzyme reaction to achieve the best hydrolysis. The hydrolysates were analyzed for degree of hydrolysis (DH), antioxidant activity (ATBS) and calcium chelating capacity. It was found that treatment with papain at 50 ºC incubation temperature, 180 min incubation time, 1.0% enzyme concentration and 7.0 pH effectively hydrolyzed the goat milk whey proteins, as determined by SDS-PAGE and measurement of nonprotein nitrogen content. Hydrolysis with Papain resulted in a significant increase in antioxidant and Ca^+ chelation property. Hence, the GMWPH may be useful for development of novel foods for infants, and the elderly osteoporosis patients to replace cow milk hydrolysates.

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