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Standardization of Optimum Conditions for Hydrolyses of Goat Milk Whey Protein with Trypsin Enzyme

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ABSTRACT

Goat milk is as close to perfect food as possible in nature and was known as "the king of milk" in the world, but the development of functional foods from goat milk has been slow compared to cow and buffalo milk. The study was conducted with an objective to develop a goat milk whey protein hydrolysate (GMWPH) with enhanced antioxidant property and better Ca^{+} chelating activity. Goat milk whey protein was digested with commercial food-grade Trypsin enzyme under various conditions of incubation temperature (30 to 70 °C), 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 (ABTS) and calcium chelating capacity. It was found that treatment with trypsin at 40 °C incubation temperature, 180 min incubation time, 1.0% enzyme concentration and 8.0 pH effectively degraded the goat milk whey proteins, as determined by SDS-PAGE and measurement of nonprotein nitrogen content. Hydrolysis with trypsin 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.

HIGHLIGHTS

- Bioactive peptides can be produced from goat milk whey proteins with trypsin enzymatic hydrolyzation under controlled conditions.
- These hydrolyzed peptides increased antioxidant properties and worked as nanotubes for calcium binding.

Keywords: Goat whey protein, Enzymatic hydrolysis, antioxidant, calcium chelating

Goat milk is as close to perfect food as possible in nature and was known as "the king of milk" in the world. Its chemical structure is amazingly similar to mother's milk (Shu *et al*., 2016). It is reflected by the 9.3 % faster increase in goat population as compared to cattle during the 20th Livestock Census of the country (Livestock Census, 2019) and the largest increase in goat milk production (58%) compared to other mammalian farm animals. Milk production of goats is likely to be much greater than in these official statistics, because of the large amounts of unreported home consumption, especially in developing countries (Kalyan *et al*., 2018).

Milk whey is an abundant by-product of the dairy industry which represents about 80–90% of milk volume and retains approximately 55% of milk nutrients (Getaneh *et al*., 2016). The goat milk 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 (Shu *et al*., 2016).

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The interesting property of α- Lactalbumin (α-La) and β-Lactaglobumin (β-Lg) whey protein is their ability to self-assemble on partial hydrolysis, which leads to formation of nanotubular structures for α-La and fibrillar aggregates for β-Lg in the presence of appropriate cation at neutral pH (Tarhan *et al*., 2014). These microstructures promise various applications in food, nanomedicine and nanotechnology. Because of its cavity, α -La nanotubes could well serve as vehicles for drugs, vitamins, enzymes and minerals or other encapsulated molecules.

Among the mineral-binding and natural antioxidant peptides derived from the digestion of milk proteins, casein phosphopeptides have been extensively investigated (Díaz-Castro *et al*., 2012), however, studies on the utilization of whey proteins as producing mineral carrier and antioxidant peptides are scarce.

In the light of above discussion, to prepare goat milk whey protein bioactive peptides and their potential applications for calcium encapsulation and natural antioxidants to develop calcium enriched functional and shelf-stable foods, the present study was designed to preparation and characterization of bioactive peptides from goat milk whey proteins.

MATERIALS AND METHODS

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 °C 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 casein curd by centrifugation at 8000 rpm/min, at 4 °C for 20 min and filtering through four layered muslin cloth. The samples were pre-frozen at -18 °C for 24 h and then placed in a freeze-dryer (SCIENTZ-10N, at NDRI, Karnal) and vacuum freeze-dried at 50 °C and 2–10 Pa to obtain freeze-dried powder samples, and stored at -20 °C until further use.

Enzymatic hydrolysis of goat milk whey proteins

Food-grade commercial protease (Trypsin) 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 (Jung *et al.,* 2016). 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), as per method followed by Nielsen *et al*. (2001). The DH was calculated according to the following equation:

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

Where, h = (serine-NH_2 - β) / α meqv / g / protein.

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

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 Salami *et al*. (2011). The $ABTS^{+}$ activity was calculated by using the following formula:

$$\text{ABTS activity (\% inhibition)} = \frac{0.7 - At_{20}}{0.7} \times 100$$

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-cresolphthalein complexone reagent using complexometric titration method as followed by Xixi *et al*. (2015) with some modifications. 250 ml 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 $p<0.05$.

RESULTS AND DISCUSSION

Optimization of incubation temperature

The hydrolysates of GMWP using trypsin enzyme were done at different incubation temperatures from 30 to 70 ºC, and hydrolysis were compared with the standard molecular weight marker using SDS-PAGE analysis (Fig. 1A).

SDS-PASE showed that the hydrolyzation of GMWP with trypsin enzyme was increased as the incubation temperature increased from 40 to 60 ºC. These findings were further confirmed with NPN method (Fig. 1B). The non-protein nitrogen was recovered up to 5.6 mg/ml at 40ºC, which was statistically similar to 50 and 60 ºC Fig 1A and 1B revealed that the hydrolyzation of GMWP was optimum at 40 ºC with trypsin enzyme. Wang *et al*. (2020) also followed 40 ºC as optimum temperature for hydrolyzation of camel and cow whey protein with trypsin.

Optimization of incubation time

The hydrolysates of GMWP using trypsin 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 trypsin enzyme was increased as the incubation time

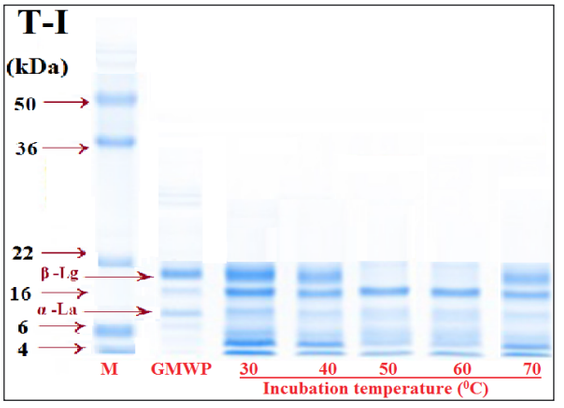

Fig. 1A: T-I = Trypsin, SDS-PAGE for different incubation temperature, M = Protein molecular marker, GMWP =g oat milk whey protein

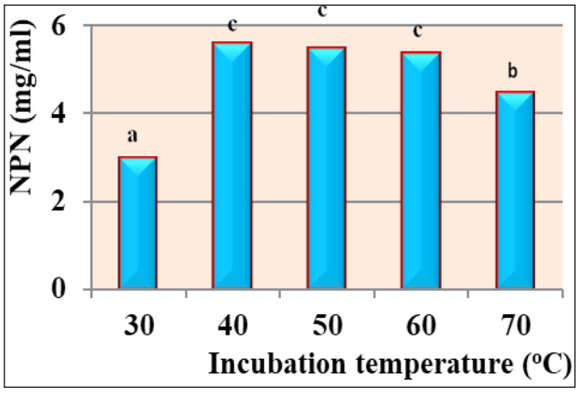

Fig. 1B: Trypsin NPN (mg/ml) for different incubation temperature, different small letters differ significantly (<0.05)

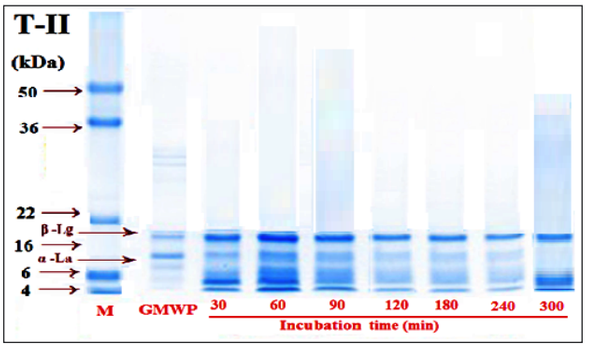

Fig. 2A: T-II = Trypsin, SDS-PAGE for different incubation time, M = Protein molecular marker, GMWP = goat milk whey protein

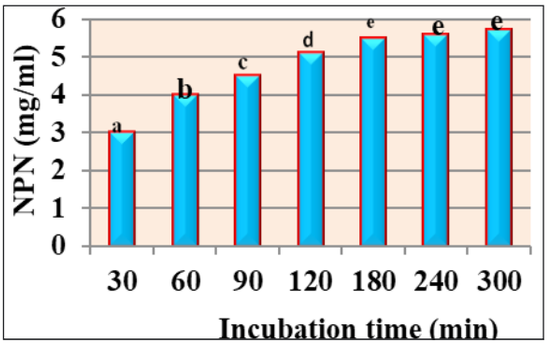

Fig. 2B: NPN (mg/ml) for different incubation time, different small letters differ significantly (<0.05)

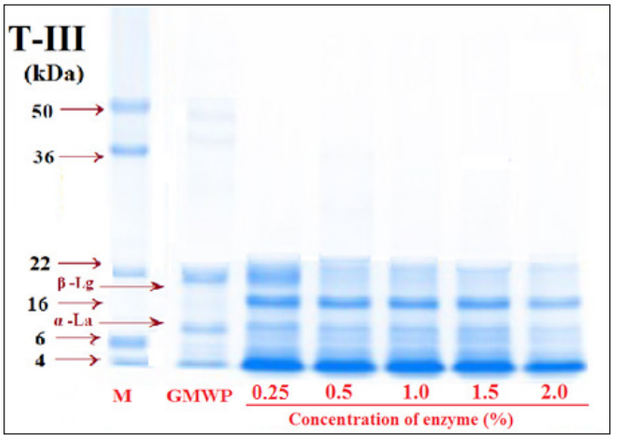

Fig. 3A: T-III = Trypsin, SDS-PAGE for different incubation Con., M = Protein molecular marker, GMWP = goat milk whey protein

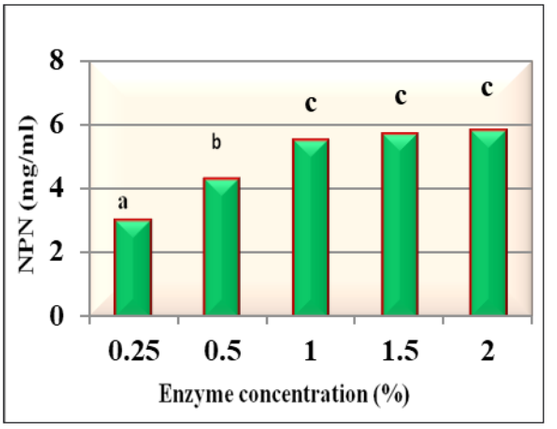

Fig. 3B: NPN (mg/ml) for different incubation Con., different small letters differ significantly (<0.05)

increased up to 180 min. These findings were further confirmed with NPN method (Fig. 2B). The non-protein nitrogen was recovered up to 5.5 mg/ml at 180 min. Fig. 2A and 2B revealed that the hydrolyzation of GMWP did not increase significantly (<0.05) after 180 min of incubation time with trypsin enzyme. Wang *et al*. (2020) also reported that camel milk whey protein was completely hydrolyzed and digested with trypsin enzyme for 180 min.

Optimization of incubation enzyme concentration

Different enzyme concentrations of trypsin enzyme from 0.25 to 2.0 percent level were used for hydrolysates of GMWP and hydrolysis were compared with the standard molecular weight marker using SDS-PAGE analysis (Fig. 3A).

SDS-PASE showed that the hydrolyzation of GMWP with trypsin enzyme was increased as the enzyme concentration increased up to 1.0 % level. These findings were further confirmed with NPN method (Fig. 3B). The non-protein nitrogen was recovered up to 5.5 mg/ml at 1.0 per cent enzyme concentration. Fig. 3A and 3B revealed that the hydrolyzation of GMWP did not increase significantly (<0.05) after 1.0% level of enzyme concentration with trypsin enzyme. An enzyme concentration of trypsin was 1% at 37°C for 4 h also considered optimum for digestion of casein by Mir *et al*. (2018) in Silk.

Optimization of incubation pH

The hydrolysates of GMWP using trypsin enzymes were done at different incubation pH levels from 6 to 10, and

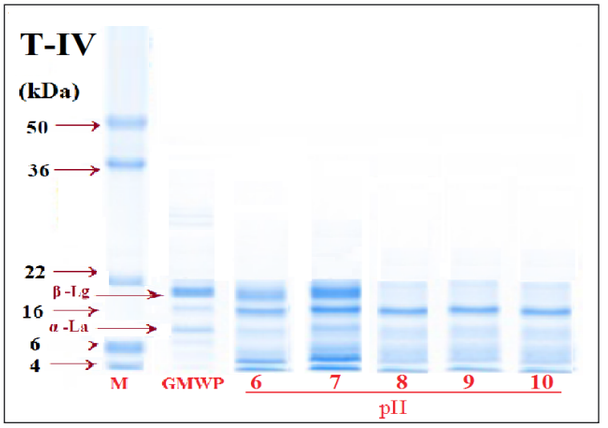

Fig. 4A: T-IV = Trypsin, SDS-PAGE for different incubation pH, M = Protein molecular marker, GMWP = goat milk whey protein

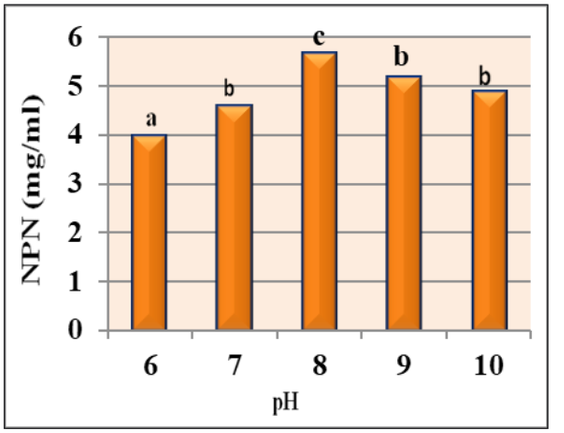

Fig. 4B: NPN (mg/ml) for different incubation pH., different small letters differ significantly (<0.05)

hydrolysis were compared with the standard molecular weight marker using SDS-PAGE analysis (Fig. 4A).

SDS-PASE showed that the hydrolyzation of GMWP powder with trypsin enzyme was highest at pH 8.0 level followed by 9.0 pH. These findings were further confirmed with NPN method (Fig. 4B).

The non-protein nitrogen was recovered 5.7 mg/ml at 8.0 pH level, which was highest. Fig 4A and 4B revealed that the hydrolyzation of GMWP was optimum at 8.0 pH level with trypsin enzyme. Mir *et al.* (2018) reported that trypsin is most active in the pH range between 7 and 9 at 37 ºC.

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 and ABTS activity of goat milk whey protein hydrolysates by trypsin were expressed in terms of percentage (%) of hydrolysis carried out under different standardized control conditions selected for different enzymes (Table 1).

DH for each enzyme increased with increase in time of hydrolysis from 1 hr to 6 hrs. It was observed that trypsin enzyme till 4 hrs (21.1%) produced peptides with increased degree of hydrolysis significantly, but after these selected times, there was no significant increase in DH of the entire enzymes. 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.

Table 1: The percent DH, ABTS and Ca^+ chelating activity of GMWPH with trypsin

Proteolysis time (Hrs)	Hydrolyzing enzymes Trypsin		
	DH (%)	ABTS	Ca+ Con. (%)
1	11.8^{a}±0.08	33.49^{a}±0.91	11.8^{a}±0.91
2	16.5^{b}±0.13	40.27^{b}±0.73	16.5^{b}±0.91
3	19.80^{c}±0.12	50.41^{c}±0.84	19.8^{c}±0.91
4	21.1^{dc}±0.09	52.68^{c}±0.69	21.1^{cd}±0.91
5	22.3^{de}±0.14	52.49^{c}±1.02	22.3^{de}±0.91
6	23.1^{e}±0.13	51.88^{c}±0.77	23.1^{e}±0.91

Mean±SE with different small letters superscripts column wise differ significantly (p≤0.05).

The ABTS radical-scavenging activity increased significantly (P<0.05) with the advancement of hydrolysis time up to 4 hrs for trypsin GMWPH. Similar reports were documented by Kumar *et al*. (2016). These findings were also in accordance with the findings of Salami *et al*. (2011) who also reported higher antioxidant activity of camel milk casein hydrolysates upon digestion with gastrointestinal enzymes.

These results indicated that the degree of hydrolyzation by trypsin enzyme treatment influences the Ca-chelating activity within 4 hrs of the obtained GMWPH. After that, DH and Ca-chelating activity did not increase significantly in trypsin treated hydrolysates. If the hydrolysis time was prolonged 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. It was concluded that hydrolysis of goat milk whey protein with trypsin 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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