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Standardization of optimum conditions for hydrolyses of goat milk whey protein with alcalase enzyme

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Abstract
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 alcalase enzyme under various conditions of incubation temperature (30 to 70 ^{0}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 alcalase at 60 ^{0}C incubation temperature, 60 min incubation time, 0.5% enzyme concentration and 7.0 pH effectively degraded the goat milk whey proteins, as determined by SDS-PAGE and measurement of nonprotein nitrogen content. Hydrolysis with alcalase resulted in a significant increase in antioxidant and Ca^{+} chelation property. Hence, the GMWPH may be useful for development of novel functional foods for infants, and the elderly osteoporosis patients to replace cow milk.

Keywords: goat whey protein, enzymatic hydrolysis, antioxidant, calcium chelating

Introduction
Goat milk is as close to perfect food as possible in nature and was known as "the king of milk" in the world [1]. Its chemical structure is amazingly similar to mother's milk [2]. It is reflected by the 9.3 % faster increase in goat population as compared to cattle during the 20th Livestock Census of the country [3]. 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 [4].
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 [5]. The goat milk whey protein peptides are rich in amino acids which are highly digestible and have positive effect on satiety and mood [6], improve morning alertness and brain-sustained attention processes [7]. 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 [8]. 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.
However, the mineral-binding and natural antioxidant peptides derived from the digestion of casein phosphopeptides have been extensively investigated [9], but 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
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 ^{0}C for 20 min and filtering through four layered muslin cloth.

The samples were pre-frozen at -18 ^{0}C for 24 h and then placed in a freeze-dryer (SCIENTZ-10N, at NDRI, Karnal) and vacuum freeze-dried at 50 ^{0}C and 2–10 Pa to obtain freeze-dried powder samples, and stored at -20 ^{0}C until further use.

Enzymatic hydrolysis of goat milk whey proteins

The optimal hydrolytic conditions were assumed to be those when *β*-lactoglobulin was degraded almost completely. Food-grade commercial protease (Alcalase) 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 [10]. 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 as per method followed by Nielsen *et al.* [11] using 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 [12]). 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).

Non protein nitrogen content

NPN contents were measured by the Folin-Lowry method [13]. 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.* [14]. The $ABTS^+$ activity was calculated by using the following formula:

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

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-cresolphthalein complexone reagent using complexometric titration method as followed by Xixi *et al.* [15]. 250ml of 2.5% (w/v) calcium-chelating peptide and 75ml 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 data obtained from different sets of each experiment were subjected to statistical analysis described by Snedecor and Cochran [16] for Analysis of Variance (ANOVA) on Completely Randomized Design (CRD) and Duncan's Multiple Range Test (DMRT) to compare the means with Standard Error (SE) performed using SPSS version 16.0 (SPSS Inc., Chicago, Release 16, 2007). Statistical significance was assumed at $p<0.05$.

Results and Discussion

Optimization of incubation temperature

The hydrolysates of GMWP using alcalase enzyme were done at different incubation temperatures from 30 to 70 ^{0}C, and hydrolysis were compared with the standard molecular weight marker using SDS-PAGE analysis (Fig. A-I) and NPN (Fig. a-I) content.

The SDS-PASE showed that the hydrolyzation of GMWP was increased as the incubation temperature increased up to 60 ^{0}C. These findings were further confirmed with NPN method also (Fig. a-I). The non-protein nitrogen at 60 ^{0}C hydrolyses was recovered up to 5.7 mg/ml. Jung *et al.* [10] also reported that GMWP treatment with alcalase hydrolyses at 60 ^{0}C resulted effectively in a significant decrease in *β*-lactoglobulin concentration (almost to nil).

Optimization of incubation time

The hydrolysates of GMWP using alcalase enzymes was 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. A-II) and NPN content (Fig. a-II).

SDS-PASE showed that the hydrolysation of GMWP with alcalase enzyme was increased as the incubation time increased up to 60 min. These findings were further confirmed with NPN method). The non-protein nitrogen was recovered up to 5.5 mg/ml at 60 min. The hydrolysation of GMWP did not increase significantly (<0.05) after 60 min of incubation time with alcalase enzyme. Jung *et al.* [10] found that the rates of GMWP hydrolysis around 30 min incubation periods with alcalase yielded maximum NPN content (approximately 6 mg/mL).

Optimization of incubation enzyme concentration

The hydrolysates of GMWP using alcalase enzymes was done at different enzyme concentrations from 0.25, to 2.0 percent and hydrolysis were compared with the standard molecular weight marker using SDS-PAGE analysis (Fig. A-III) and NPN (Fig. a-III) content. SDS-PASE showed that the hydrolysation of GMWP with alcalase enzyme was increased as the enzyme concentration increased up to 0.5 %. These findings were further confirmed with NPN method (Fig. a-III). The non-protein nitrogen was recovered around 5.4 mg/ml at 0.5 per cent enzyme concentration. The similar reports were documented by Jung *et al.* [10]. They reported that hydrolysis of goat milk proteins by 0.4% alcalase at 60 °C for 30 min was found to be the most effective, resulting in a

significant reduction in the amounts of *β*-lactoglobulin in the GMPH according to SDS-PAGE analysis.

Optimization of incubation pH

The hydrolysates of GMWP using alcalase enzymes was done at different pH levels from 6 to 10, and hydrolysis were compared with the standard molecular weight marker using SDS-PAGE analysis (Fig. A-IV) and NPN content (Fig. a-IV).

SDS-PASE showed that the hydrolysation of GMWP with alcalase enzyme was not increased as the pH of reaction increased after 7.00. These findings were further confirmed with NPN method. The maximum NPN was obtained at pH 9.0 (5.7 mg/L). This is similar to previous studies on Pacific whiting (Merluccius products) solid waste that showed maximum % NPN at pH 9.5 [17]. However, at this pH 9.0, the hydrolysis solution showed a colour change from brown to blackish with urea smell. This was indicative of very extensive protein degradation to a level of amino acid breakdown. Previous studies have however shown that amino acids do not exhibit very good antioxidative properties thus for this study that involved antioxidative function analysis, it was a requirement to stop the proteolysis at least at a dipeptide [18]. Consequently, the pH (7.0) with the next highest NPN (5.6 mg/ml) was selected.

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 alcalsase were expressed in terms of percentage (%) of hydrolysis and ABTS (Table 1).

However, DH (%) for alcalase enzyme increased with increase in time of hydrolysis from 30 to 300 min, but it was observed that alcalase enzyme 60 min (29.14%) produced peptides with increased degree of hydrolysis significantly, but after these selected times, there was no significant increase in DH. Similar reports were also documented by Kumar *et al.* [19]. The reduction in hydrolysis rate over time may indicate the decreased availability of cleavable peptide bonds within the substrate [19].

The ABTS radical-scavenging activity increased significantly ($P<0.05$) with the advancement of hydrolysis time up to 60 min for alcalase GMWPH. These findings were also in accordance with the findings of Jrad *et al.* [20] and Salami *et al.* [14] 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 alcalase enzyme treatment influences the Ca-chelating activity within 60 min of the obtained GMWPH. If the hydrolysis time was prolonged after 60 min, 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.* [15] 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 alcalase 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.

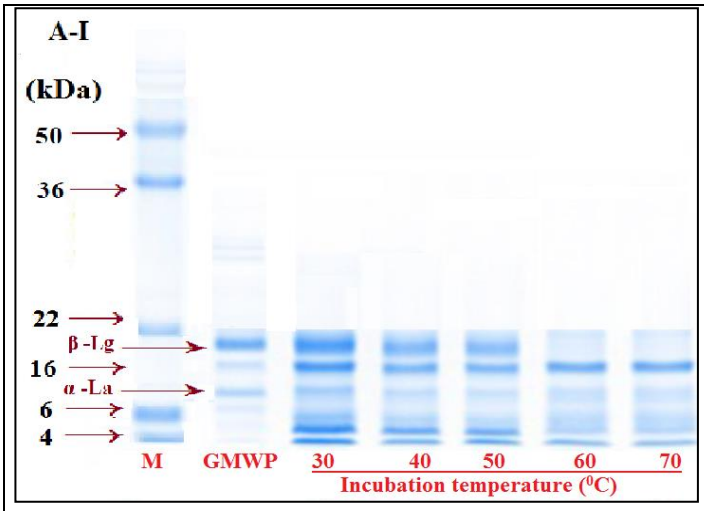

Fig A-1: Optimization of incubation temperature of goat milk whey proteins hydrolyzed with Alcalase using SDS-PAGE.

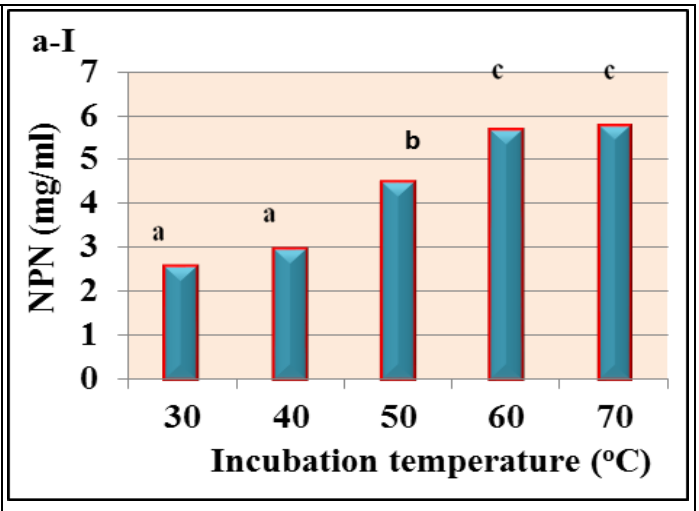

Fig a-I: NPN (mg/ml) at different incubation temperature, different small letters differ significantly (<0.05).

β-Lg= Bita-Lactoglobulin, *α*-La= Lactalbumin, M= Protein molecular marker, GMWP=goat milk whey protein.

Fig A-I and Fig a-I: Optimization of incubation temperature of goat milk whey proteins hydrolyzed with Alcalase.

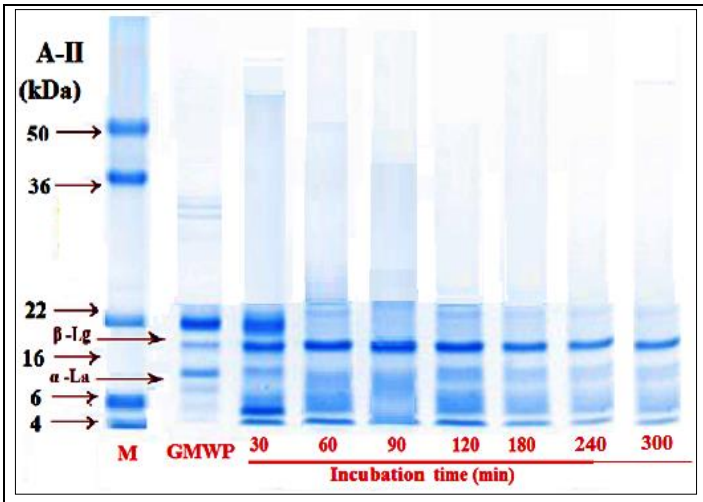

Fig A-II: Optimization of incubation time of goat milk whey proteins hydrolyzed with Alcalase using SDS-PAGE.

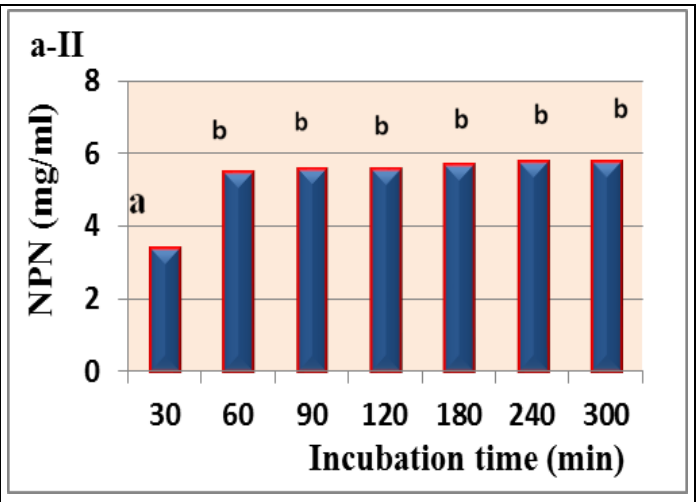

Fig a-II: Alcalase NPN (mg/ml) at different incubation time, different small letters differ significantly (<0.05).

β-Lg= Bita-Lactoglobulin, *α*-La= Lactalbumin, M= Protein molecular marker, GMWP=goat milk whey protein.

Fig A-II and Fig a-II: Optimization of incubation time of goat milk whey proteins hydrolyzed with Alcalase.

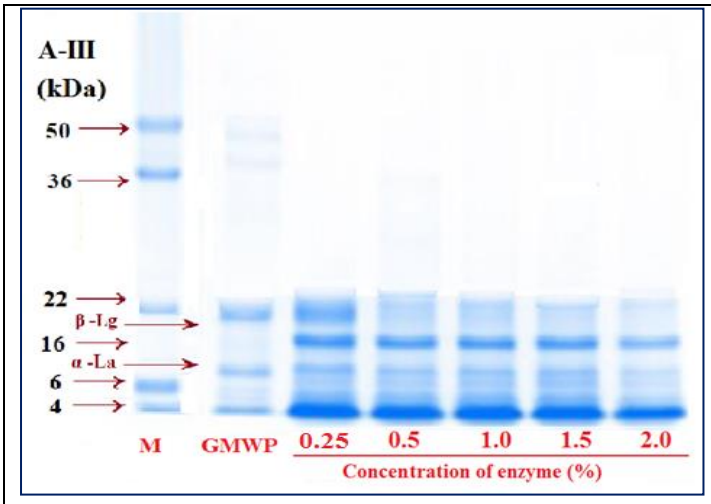

Fig A-III: Optimization of enzymes concentration of goat milk whey proteins hydrolyzed with alcalase using SDS-PAGE.

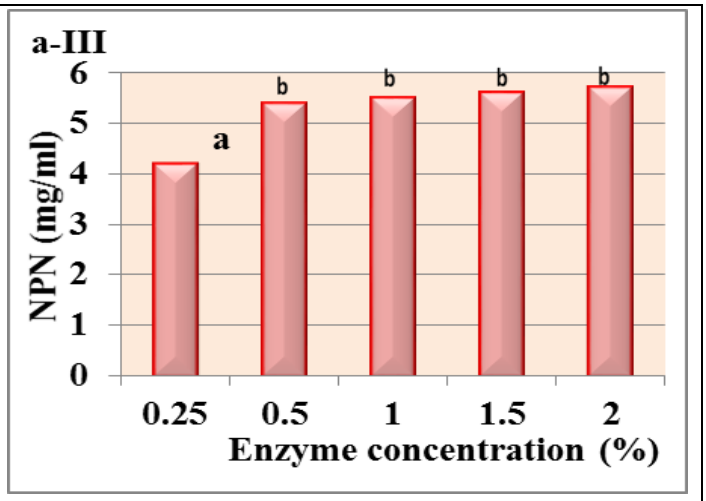

Fig a-III: Alcalase NPN (mg/ml) at different enzyme concentration, different small letters differ significantly (<0.05).

β-Lg= Bita-Lactoglobulin, *α*-La= Lactalbumin, M= Protein molecular marker, GMWP=goat milk whey protein.

Fig A-III and Fig a-III: Optimization of enzyme concentration for goat milk whey proteins hydrolyzed with Alcalase.

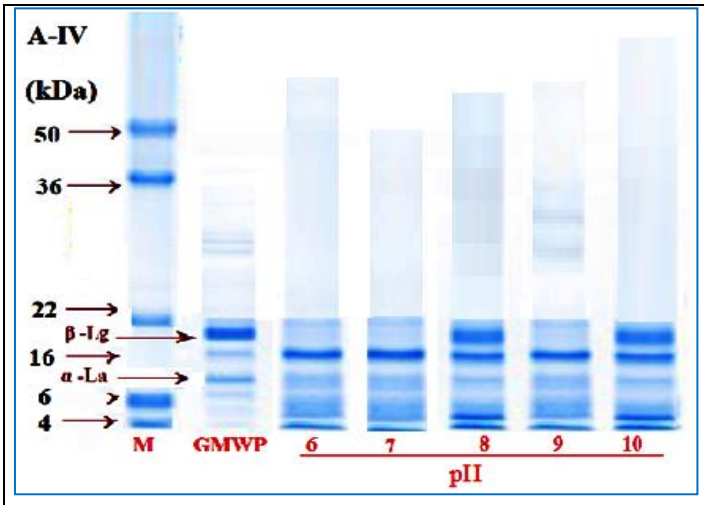

Fig A-IV: Optimization of pH for goat milk whey proteins hydrolyzed with Alcalase using SDS-PAGE.

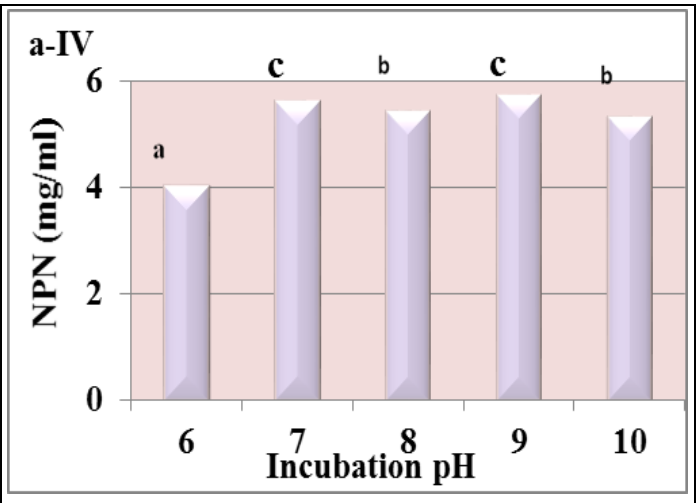

Fig a-IV: Alcalase NPN (mg/ml) at different pH, different small letters differ significantly (<0.05).

β-Lg= Bita-Lactoglobulin, *α*-La= Lactalbumin, M= Protein molecular marker, GMWP=goat milk whey protein.

Fig A-IV and Fig a-IV: Optimization of incubation pH of goat milk whey proteins hydrolyzed with Alcalase.

Table 1: The percent DH, ABTS and Ca^{+} chelating activity of GMWPH with alcalase.

Proteolysis time (min)	Hydrolyzing enzymes alcalase		
	DH (%)	ABTS	Ca+ Con. (%)
30	21.32^{a}±0.08	67.79^{b}±0.87	26.70^{a}±0.91
60	29.14^{b}±0.12	67.04^{b}±0.98	33.28^{b}±0.87
90	29.51^{b}±0.13	64.87^{ab}±1.57	34.41^{b}±0.82
120	30.13^{bc}±0.14	63.98^{a}±1.83	34.92^{bc}±0.77
150	30.4^{bc}±0.09	62.99^{a}±2.01	35.31^{c}±0.1.21
300	31.5^{c}±1.76	62.54^{a}±1.76	35.42^{c}±0.94

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

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