Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

JEZS Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(4): 1012-1015

Received: 21-05-2020
Accepted: 15-07-2020

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Evaluation of anthelmintic activity of bioactive peptides derived from enzyme treated goat milk Casein

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Abstract
Goat milk is particularly rich in protein content leading to profound nutritional significance. Majority of biological activities of milk is attributed to the milk protein casein and the bioactive peptides derived from it. Majority of the drugs available in the market to treat parasitic worm infections, have common side effects leading to limitations of their use. The present work was designed with the aim to evaluate the anthelmintic activity of bioactive peptides derived from goat milk casein upon treatment with proteolytic enzymes Trypsin, Chymotrypsin and Pepsin. From goat milk, casein was isolated and treated with different enzymes separately. Bioactive peptides obtained were analysed for their anthelmintic effects using in-vitro method. Albendazole was used as standard for the experimental purpose. As compared to the standard, only peptic and tryptic hydrolysates showed better anthelmintic activity. The data obtained from the present study hence proved that bioactive peptides released by peptic and tryptic digestion of goat milk casein contains significant health effects thereby suggesting their potential use in future as neutraceuticals for the treatment of helmintic infections without having any side effects.

Keywords: Bioactive peptides, Anthelmintic, Trypsin, Chymotrypsin, Pepsin, Albendazole, Neutraceuticals

Introduction
Milk is a well-balanced source of nutrients having biological activities that influence various biochemical and physiological processes of the body in a positive manner. Biological effects of the milk are mainly due to its protein content and associated peptides. The benefits of milk in preventing various infections have been recognized for thousands of years. Much of this activity has been attributed to antibodies, but the role of milk sugars and milk proteins as bioactive agents is only recently being recognized. Milk contains various components with physiological functionality [1].
Goat milk has high protein content but lower in fat similar to human milk. It is much beneficial as compared to milk of other animal species because of less allergic nature, naturally homogenized form, easily digestible and rarely causes lactose intolerance. It contains lower amount of alpha casein (mainly responsible for milk allergy) and high in beta casein fraction as compared to cow milk. Beta casein of goat milk is having greater solubilisation than cow milk [2]. The curd of goat milk is also weaker which directly influences the digestibility in the gastrointestinal tract.
Bioactive peptides are the specific protein fragments having positive effect on different body functions and conditions, thereby influencing overall health status [3]. According to their functional properties, bioactive peptides may be classified as antimicrobial, antithrombotic, antihypertensive, opioid agonist, opioid antagonist, immuno modulatory, mineral binding, antioxidative and anti-inflammatory etc. Milk protein casein is one of the richest source of these bioactive peptides [4, 5]. There are several methods to produce bioactive peptides from milk protein casein and the most commonly used approach is by enzymatic digestion using different proteolytic enzymes either alone or in combination. After their release from milk, these peptides acts as modulators of many regulatory biological processes thus exhibiting wide array of biological and physiological effects [6]. The major protein in goat milk is beta casein. Bioactive peptides derived from goat casein by treatment using purified enzymes presented significant antihypertensive and Immuno modulatory activities [7].

A previous study demonstrated that goat milk protein on treatment with pepsin generated multiple soluble peptides with remarkable radical scavenging activities [8].

Helminthic disease have a worldwide distribution affecting billions of people in endemic areas and can result in serious clinical complications. Anthelmintic are drugs that either kill or expel these parasitic worms. Majority of these drugs available presently are having common side effects leading to limitations in their use. Previous studies have shown that milk-fed ruminants were less susceptible to gastrointestinal parasites compared with young animals that were given solid feed in the diet. The active component appears to be associated with milk proteins fractions. The lack of effect when milk and solid feed are ingested together may result from protein attaching to feed particles in preference to the larvae, leading to limitation s in practical applications of milk proteins as anti-parasitic agents in ruminants consuming solid feed. Camel’s milk offers a practical and sustainable resource to tackle widespread gastro-intestinal sheep worms in low input production systems [9].

Though significant work has been done on the bioactive peptides obtained from goat casein, but the peptides used in earlier studies were produced by microbial culture derived enzymes and moreover anthelmintic activity of goat casein derived peptides has been not studied till date. This lead to the foundation for the present study to investigate the presence of anthelmintic bioactive peptides derived from goat milk casein upon treatment with purified proteolytic enzymes under conditions simulating the human digestive tract.

Materials and Methods

Trypsin, ACE and HHL are from Sigma-Aldrich; while all the other chemicals used were of analytical grade.

1. Preparation of Casein:

Goat milk sample were collected from local breeds of goat in the vicinity of Suddhowala, Dehradun, India. Casein was prepared from milk using isoelectric-precipitation method. 500ml milk, immediately after collection, was defatted by centrifuging twice at 5000gfor 20 min at 4°C. The filtrate was diluted with equal volume of double distilled water (DDW); pH adjusted to 4.6 with 1 N HCl and the mixture was stirred for 20 min. The precipitate so formed was separated by filtration through four layer of cheese-cloth, washed, solubilized in distilled water at pH 7.0 (equal to initial volume of milk) with 1N NaOH, reprecipitated and washed 3-4 times with distilled water. The wet casein after thorough washing with distilled water was dried at room temperature for 28 hours to obtain a dry powder.

2. Preparation of Sodium Caseinate

Dried casein was solubilised in distilled water (3gm/50 ml) by continuous stirring with the help of magnetic stirrer and simultaneous addition of 0.1N NaOH drop wise so as to obtain pH of this homogenous solution to 7.2. The final volume was made up to 100ml with double distilled water. The solution of sodium caseinate was stored at 4ºC, till it was processed for further studies. The concentration of protein in various samples was estimated by using the method of Lowry’s method [10].

3. Treatment with Proteolytic enzyme

Casein prepared was treated with Trypsin, Chymotrypsin & Pepsin enzymes according to the method suggested by Abubakar *et al.* [11] & Pihlanto-leppala *et al.* [12].

0.125g of casein was taken (containing 100mg of protein) and dissolved in 20ml of the respective buffer (as per the enzyme used – table 1). To this 100µl of 1% enzyme solution (100mg in 10ml of same buffer as used for casein) was added to get substrate to enzyme ratio of 100:1 and stirred at temperature defined for the particular enzyme treatment for 4 hours (as it’s the time taken for the food to get digested in our gastrointestinal tract thereby simulating the physiological conditions). After completion of the incubation period, enzyme activity was stopped by heating at 100ºC for 5 min. The supernatant and pellet was separated by centrifugation at 10,000rpm for 20 min at 5º C and supernatant stored at 4º C in a deep freezer.

Table 1: Conditions employed for hydrolysis of casein by different enzymes

Enzyme	Buffer Used	pH	Incubation Temperature (ºC)
Chymotrypsin	0.02M Ammonium Acetate	8	25
Trypsin	0.05M Tris HCl	2	37
Pepsin	0.05M HCl	2	37

4. Estimation of Partial Hydrolysis of Protein by Hull’s method [13]

A stock solution of tyrosine of 0.2mg/ml was prepared. Different concentrations ranging from 100 µg to 1000 µg were taken. The volume was made to 6 ml with DDW. To these 10 ml of 0.72N TCA was added and kept at 37°Cfor 15 min. In 5 ml of this aliquot, 15% sodium carbonate in 0.1 N NaOH-Copper Sulphate solutions was added and mixed thoroughly. Finally 3ml of Folin's Reagent was added. This mixture was kept in dark for 5-10 min for color development. The blue color thus developed was measured at 650 nm. A standard curve plotting tyrosine concentration against O.D. was prepared.

For quantification of enzyme hydrolysates, in place of different concentrations of tyrosine, supernatant samples from different enzymatic treatments were taken.

5. Assay of Anthelmintic Activity

Anthelmintic activity of hydrolysates were estimated by using the method of Ajaiyeoba *et al.*, [14]. The assay was performed in-vitro using earthworm of 6-8cm in size. Test solution here is the casein hydrolysates obtained after treating casein with different enzymes. Two earthworms of approximately equal size were placed in 10 small petri plates containing: 10ml of different enzyme hydrolysates in duplicate (6 petri plates), albendazole (10mg/ml in double distilled water) as reference standard (1 petri plate); and 10ml of each buffer solutions used as negative controls (3 petri plates). All the samples and solutions were prepared freshly just before the use.

Two different types of observations were recorded for each petri-plate: Time taken for paralysis was noted when no apparent movement of any sort could be observed; after that, time taken for death of worms was recorded after ascertaining that worms did not move when shaken vigorously.

Results and Discussions

The dry weight of casein was found to be 2.66gm/100ml of goat milk. The value obtained was near about the cited value (3gm/100ml of milk as reported by Saini & Gill [15]). The total protein content of casein was found to be 370µg/10µlof sodium caseinate.

Degree of Hydrolysis (DH): DH measures the content of peptide bonds cleaved in the substrate by a proteolytic agent; higher the DH, the higher the content of cleaved amino groups, thereby affecting the biological activity of hydrolysates. Therefore, the biological activity of peptides depends on the substrate, enzyme specificity and hydrolysis conditions [16, 17, 18].

DH was found maximum on treatment with pepsin, followed by trypsin. This shows that pepsin utilized more protein as substrate to cause hydrolysis as compared to other two enzymes. Chymotrypsin treatment yields minimum DH (Table 2), indicating resistance of goat casein to this enzyme.

Table 2: Degree of hydrolysis of goat milk casein

Enzyme used	% Hydrolysis
Chymotrypsin	20.35
Trypsin	28.56
Pepsin	38.33

Assay of Anthelmintic Activity: Observation were made for the anthelmintic activity of casein hydrolysates (i.e. time taken for paralysis and time taken for death of worms) as given in the Table 3. Peptic hydrolysates showed excellent anthelmintic effects as compared with the reference standard, followed by tryptic hydrolysates (Figure 1 & 2). Chymotryptic hydrolysates also showed anthelmintic activity but lower than the reference standard (Figure 3).

Result obtained were in accordance with the DH by different enzymes, indicating that the peptides responsible for the anthelmintic activity were released considerably only after digestion with trypsin and pepsin.

Table 3a: Anthelmintic activity of chymotryptic hydrolysates of casein

	Time Taken For Paralysis (In min)	Time Taken For Death (In min)
Control (Ammonium Acetate Buffer)	28.53±3.07	31.33±2.02
Standard (Albendazole)	18±0.30	29.82±0.42
Casein Hydrolysate	34.83±2.97	41±0.58

Table 3b: Anthelmintic activity of tryptic hydrolysates of casein

	Time Taken For Paralysis (In min)	Time Taken For Death (In min)
Control (Tris Buffer)	2±0.25	6.28±0.58
Standard(Albendazole)	18.33±0.62	29.27±0.64
Casein Hydrolysate	15.32±0.64	24.03±0.86

Table 3c: Anthelmintic activity of peptic hydrolysates of casein

	Time Taken For Paralysis (In min)	Time Taken For Death (In min)
Control (HCl Buffer)	1.01±0.30	2.17±0.53
Standard(Albendazole)	18.15±1.22	29.07±1.09
Casein Hydrolysate	5.22±1.22	8.10±1.24

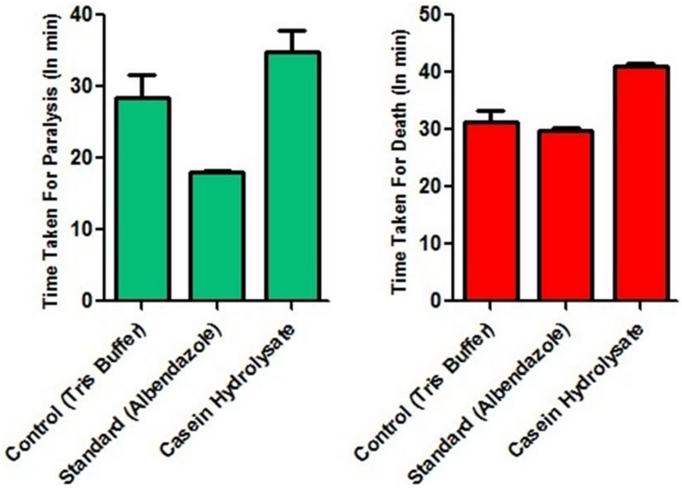

Fig 1: Histogram showing anthelmintic effect of chymotryptic hydrolysates of casein

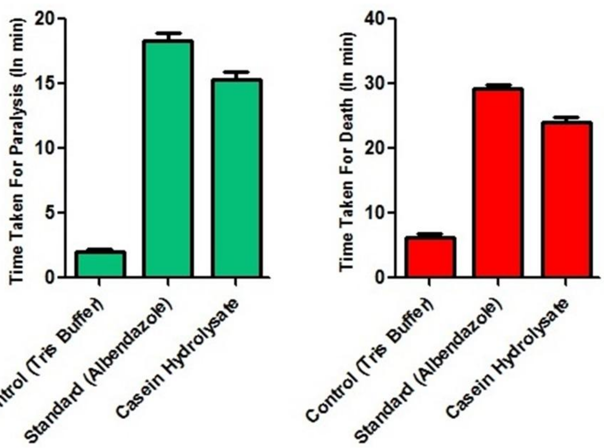

Fig 2: Histogram showing anthelmintic effect of tryptic hydrolysates of casein

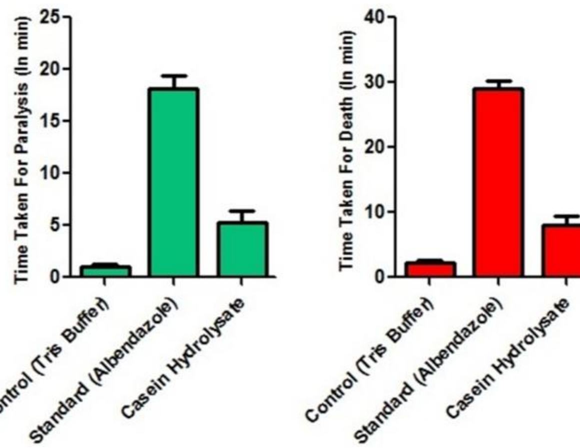

Fig 3: Histogram showing anthelmintic effect of peptic hydrolysates of casein

Previous in-vitro experiments has also demonstrated a direct detrimental effect of milk on the motility of earth worms [19] where the active fraction which appeared to influence the anthelmintic activity was protein and the protein fraction that influences this activity is the casein.

Conclusions
From the data obtained with the present study, it could be concluded that peptic and tryptic hydrolysates of goat milk casein showed significant anthelmintic activity. This can be utilized for exploring the potential applications of goat casein derived peptides for incorporation in pharmaceutical and neutraceutical preparations for delivering specific health benefits.

Acknowledgments
The author is thankful to the Dolphin (PG Institute of Biomedical & Natural Sciences, Dehradun for providing all the necessary chemicals and laboratory facility for this research work.

Conflict of interest
All the authors have no conflict of interest to declare.

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