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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(9): 2347-2352

www.thepharmajournal.com
Received: 20-06-2023
Accepted: 24-07-2023

Nagamani A
Assistant Professor, Extension Education Unit, Naganahalli, Mysuru, Karnataka, India

Rajanna M
Assistant Professor, Extension Education Unit, Naganahalli, Mysuru, Karnataka, India

Corresponding Author:
Nagamani A
Assistant Professor, Extension Education Unit, Naganahalli, Mysuru, Karnataka, India

Immunomodulatory bio-active peptides and their nutritional assessment in weaning foods fed to mice

Nagamani A and Rajanna M

Abstract
Bio-active peptides (BAPs) play a significant role on various physiological functions. Milk is the best source for bio-active peptides. In order to harvest their immunomodulatory effects they need to be isolated. Studies were conducted to isolate, BAPs from buffalo milk proteins through enzymatic hydrolysis, hydrolysates were nutritionally evaluated incorporated into weaning foods to explore their phagocytic activity. BAPs from neutrase hydrolysates of α-casein and β-lactoglobulin showed higher yield as also protein percentage when compared to BAPs from trypsin hydrolysate. Bio-active peptides (BAPs) showing maximum phagocytic activity were incorporated at 5 and 10 percent levels (dry blended) into the weaning foods. Nutritional evaluation of the formulated weaning foods {α-casein BAPs (F_1 and F_2)} showed low PER and FER values at 5 percent level compared to 10 percent level. The BAPs produced from neutrase hydrolyzed α-casein and β-Lactoglobulin showed increased number of macrophages (4.3 and 1.8 percent respectively) and phagocytic index (4.39 and 4.01 respectively), when compared to BAPs of trypsin hydrolysates. Animal experimentation concluded that, the BAPs of α-casein are more promising in enhancing the immunomodulation in mice as it showed higher macrophage percentage (76%) and higher total leucocyte count (TLcC) compared to 5 percent level and control.

Keywords: Bio-active peptides, protein efficiency ratio, feed efficiency ratio, macrophage, total leucocyte count

Introduction
The components of milk provide nutrients, immunological protection and act as bio-active agents such as proteins and peptides, carbohydrates, lipids and glycolipids and other minor bio-molecules. Proteins, are the major components of milk synthesized in the mammary gland, 60% of the amino acids used to build the proteins are derived from the cow's diet. Milk proteins have major importance as superior quality nutrients, they also perform the protective function. Bio-active peptides are the protein derived peptides that are defined as sequences of amino acids which are inactive within the native protein, but display specific properties once they are released by the enzymatic hydrolysis which are resistant to further proteolytic digestion. CPPs are the bio-active peptides (BAPs) obtained by urea fractionation through enzymatic hydrolysis are phosphorylated casein-derived peptides which have phosphorous bound via monoester linkages to seryl residues Ser (p)- Ser (p)- Ser (p)- Glu- Glu [Mellander, 1950] [7]. They are obtained by enzymatic hydrolysis of milk proteins and can be easily incorporated into foods. Studies of Grimble and Silk (1989) [5] revealed that the peptides may be absorbed slightly better and more quickly than amino acids or whole proteins in several human beings and animals. The present study was aimed at isolation of bio-active peptides from buffalo milk proteins, their incorporation into weaning foods to assess their acceptability by lab animals and nutritional evaluation of weaning foods.

Material and Methods
Buffalo milk samples were collected from Milk Co-operative Society. Casein hydrolysates were prepared and fractionated as per the procedure of Hipp *et al*., (1952) [6] using commercially available enzymes were used and whey protein fractions were isolated by adapting the method of Aschaffenburg and Drewery (1955) [4]. The hydrolysates thus obtained were the bio-active peptides. The total ash and moisture content of BAPs obtained from hydrolyzed casein and whey protein fractions were evaluated as per the procedure of AOAC (1984) [2] whereas, nitrogen and protein content by micro-kjeldhal method as per the procedure of AOAC (1980) [3]. Similarly, weaning foods incorporated with different levels of BAPs (5 and 10%) were analyzed for nitrogen and protein content by micro-kjeldhal method.

The total ash, total solid and moisture content were determined gravimetrically as per the reference procedure indicated above. Phagocytosis for mice peritoneal macrophages were carried out by adapting the procedure of Shalini Jain (2007) [8]. The number of macrophages produced after addition of BAPs isolated from hydrolyzed casein and whey protein fractions were counted and expressed in percentage. Bio-active peptides (BAPs) showing maximum phagocytic activity were incorporated at 5 and 10 percent levels into the weaning foods. Weaning foods (Lactogen) were procured from the local market. Five groups of weaning foods were formulated as;

- F_0: Control group containing weaning food with unhydrolyzed casein
- F_1: Weaning food containing neutrase hydrolyzed α-Casein BAPs incorporated at 5% level
- F_2: Weaning food containing neutrase hydrolyzed α-Casein BAPs incorporated at 10% level
- F_3: Weaning food containing neutrase hydrolyzed β-Lactoglobulin BAPs incorporated at 5% level
- F_4: Weaning food containing neutrase hydrolyzed β-Lactoglobulin BAPs incorporated at 10% level

Maintenance of Laboratory animals: Thirty Laboratory albino weanling mice aged 21 days, having an initial body weight ranging from 13-18g were procured from Small Animal House of Karnataka Veterinary Animal Fisheries Sciences University, Hebbal, Bengaluru-24 & conducted experiment as per institution animal ethical committee. They were segregated into five different groups having 6 mice in each group which were maintained in individual cages. Mice were segregated into different groups based on the treatment given. Food and water were given *ad libidum*. After allowing an initial period of 3 days for adaptation, formulated weaning foods were fed to different groups of mice maintained separately for a period of 30 days. The weight of each mouse was noted in every group at 10 days interval starting from 0 day following 10, 20 and 30 days. Average weight of the mice was finally taken to evaluate the nutritional parameters.

Results & Discussion

Compositional characteristics of BAPs: The BAPs isolated from hydrolyzates of casein and whey protein fractions using neutrase and trypsin enzyme were analyzed for nitrogen, protein, moisture and total ash content (Table-1 & Table – 2), the BAPs obtained from hydrolysis of α-casein using neutrase enzyme contain more nitrogen and protein (12.51 and 79.80 percent) when compared to trypsin enzyme (11.96 and 76.30 percent) respectively. Similarly, in case of BAPs obtained from hydrolysis of β-casein using neutrase enzyme also contain more nitrogen and protein (11.45 and 73.10 percent) when compared with trypsin enzyme (11.28 and 72.00 percent) respectively. The moisture and ash content of BAPs obtained from neutrase hydrolyzed α-casein was 0.20 and 15.00 percent respectively, the corresponding values for trypsin enzyme was 0.23 and 16.05 percent respectively. Similarly, moisture and ash content of BAPs obtained from neutrase hydrolyzed β-casein was 0.30 and 20.50 percent and for trypsin enzyme the corresponding values were 0.32 and 21.50 percent respectively.

Table 1: Compositional characteristics of Bio-active peptides (BAPs) from enzymatic hydrolysis of casein fractions

Source of BAPs	Proteolytic Enzymes	Nitrogen	Protein	Moisture	Ash
		Percent			
α-casein	Neutrase*	12.51	79.80	0.20	15.00
	Trypsin**	11.96	76.30	0.23	16.05
β-casein	Neutrase*	11.45	73.10	0.30	20.50
	Trypsin**	11.28	72.00	0.32	21.50

Table 2: Compositional characteristics of Bio-active peptides (BAPs) from enzymatic hydrolysis of whey protein fractions

Source of BAPs	Proteolytic Enzymes	Nitrogen	Protein	Moisture	Ash
		Percent			
α-lactalbumin	Neutrase*	7.92	50.55	0.26	21.45
	Trypsin**	7.70	49.17	0.28	22.00
β-lactoglobulin	Neutrase*	8.75	55.79	0.21	21.00
	Trypsin**	8.17	52.15	0.22	21.50

All values are average of three trials
* Hydrolysis carried out at an optimum pH 7.5 and temperature 45 °C
** Hydrolysis carried out at an optimum pH 8.0 and temperature 40 °C

Analysis of Chemical Composition of formulated weaning foods: The chemical composition (Table-3) of weaning foods incorporated with neutrase hydrolyzed BAPs of α-casein and β-Lactoglobulin at 5 and 10 percent levels showed nitrogen values in the range of 9.29 to 12.88%, protein content in the range 59.31 to 82.17%, total solids from 93.20 to 95.20%, moisture varied from 4.8 to 6.8% and ash content from 1.9 to 2.7%. It is very much clear from the study that, the BAPs of α-casein and β-lactoglobulin incorporated weaning foods at 5 and 10% levels resulted in higher percentage of nitrogen and protein compared to control sample. The total solid and ash content of weaning food incorporated with BAPs of α-casein and β-lactoglobulin at 10% level was higher compared to weaning food incorporated with BAPs of corresponding fractions at 5% level and control sample. This is mainly due to increased level of incorporation which pushed the level of total solids and ash content in the weaning foods with a concomitant drop in moisture percentage when compared to the composition of weaning food incorporated BAPs at 5% level.

Table 3: Chemical Composition* of weaning foods incorporated with BAPs

Type of weaning food	Level of incorporation (%)	Nitrogen	Protein	Total solids	Moisture	Ash
		Percent				
F_0	0	9.29	59.31	93.2	6.8	1.9
F_1	5	11.16	71.20	94.6	5.4	2.3
F_2	10	12.88	82.17	95.2	4.8	2.7
F_3	5	11.41	72.79	94.4	5.6	2.1
F_4	10	12.15	77.53	95.1	4.9	2.6

* All values are expressed in percentage and average of three trials

- F_0: Control group containing weaning food with unhydrolyzed casein
- F_1:Weaning food containing neutrase hydrolyzed α-Casein BAPs incorporated at 5% level
- F_2:Weaning food containing neutrase hydrolyzed α-Casein BAPs incorporated at 10% level
- F_3:Weaning food containing neutrase hydrolyzed β-Lactoglobulin BAPs incorporated at 5% level
- F_4:Weaning food containing neutrase hydrolyzed β-Lactoglobulin BAPs incorporated at 10% level

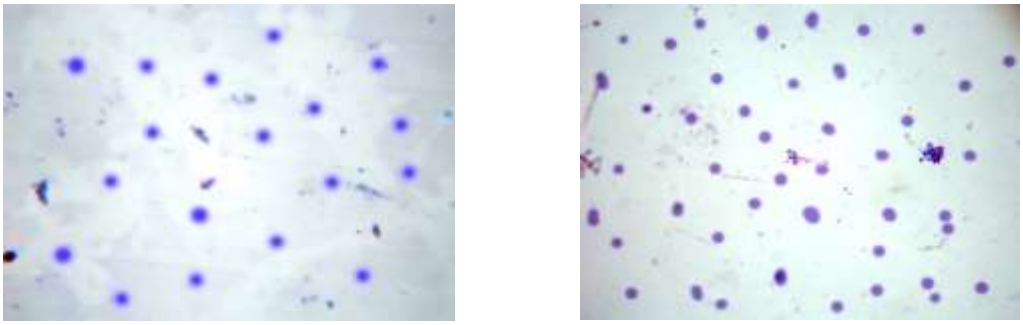
Assay for Macrophages: The BAPs obtained from hydrolysates of casein and whey protein fractions were analyzed for phagocytic activity (Table 4; Fig. 1 & 2). BAPs produced from neutrase hydrolyzed α-casein showed higher macrophage percentage (76%), when compared to trypsin enzyme (63%). Similarly, the BAPs obtained from neutrase hydrolyze β-Lactoglobulin showed higher macrophage percentage (58%), when compared to trypsin hydrolyzed whey protein fractions (52%). The increased macrophage percentage is due to the reason that α-casein contains the residues (Thr-Thr-Met-Pro-Leu-Trp) in the region of 194-199 which stimulate the immune system that are responsible for phagocytosis of peritoneal macrophages in mice. The increase in the TLcC values in case of α-casein is due to reason that, it contains the residues (Thr-Thr-Met-Pro-Leu-Trp) in the region of 194-199 which contributes to the hike in level of leucocytes. α-casein and β-lactoglobulin have substantial amounts of glutamylcysteine groups which supply the amino acid precursors (L-glutamate, L-cysteine and Glycine) necessary for the formation of glutathione (L-glutamylcysteinylglycine), which is responsible for immune-enhancing effect. Cysteine, a sulphur containing amino acid is the preferred substrate for synthesis of Glutathione and enhancement of immune system. Antigen presenting cells such as, macrophages prefer cysteine for glutathione synthesis which is a pre-requisite to initiate the immune response. Hence, the BAPs of α-casein are more promising in immune enhancement process. It is inferred that, the increase in macrophage number was observed in the weaning foods added with BAPs of neutrase hydrolyzed α-casein (among casein fractions) and β-lactoglobulin (among whey protein fractions) as compared with BAPs derived from trypsin.

Table 4: Enumeration of macrophages in the peritoneal fluid of mice

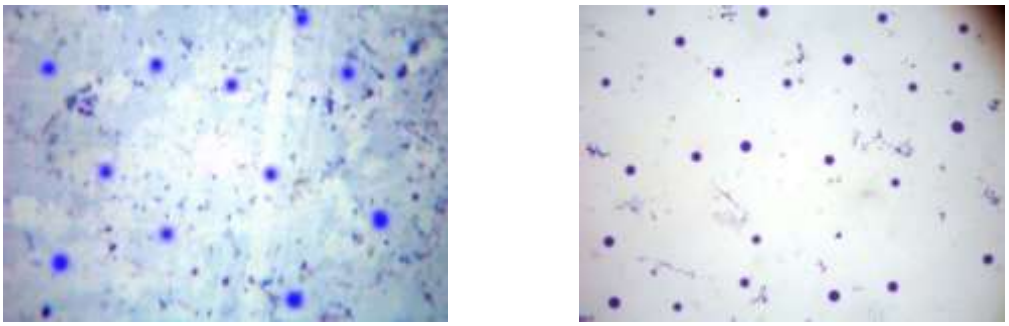
Source of BAPs	Number of Macrophages (Percent)	
	Neutrase	Trypsin
α- casein	76	63
β- casein	54	48
α-lactalbumin	43	39
β- lactoglobulin	58	52
CD ($p \leq 0.05$)	0.08	

All values are average of 6 trials

Before After

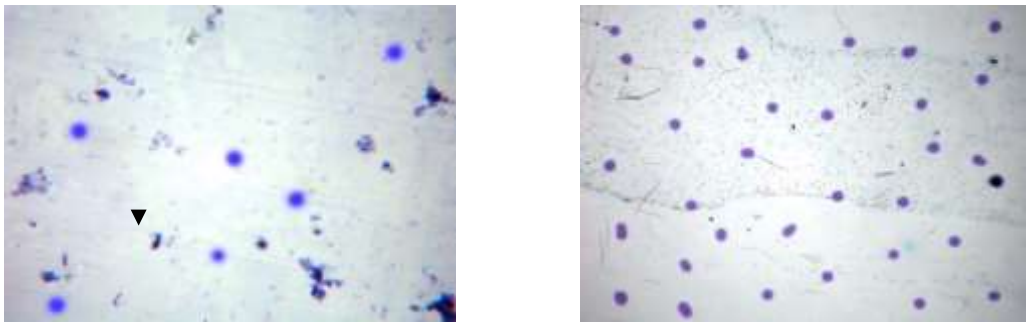


a. Neutrase hydrolyzed α-Casein BAPs

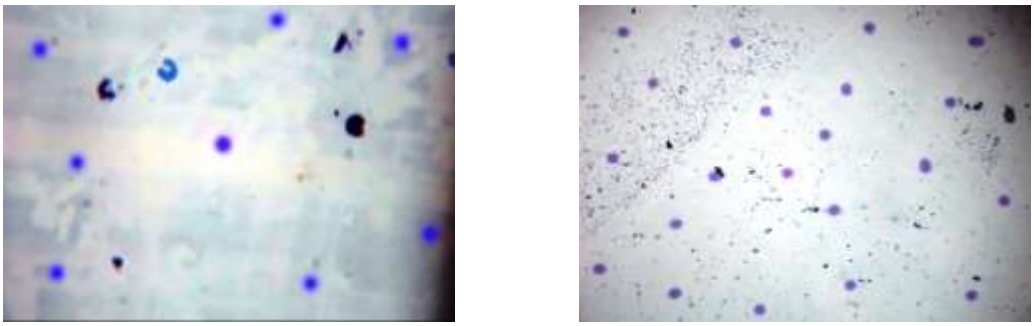


b. Trypsin hydrolyzed α-Casein BAPs

Before After



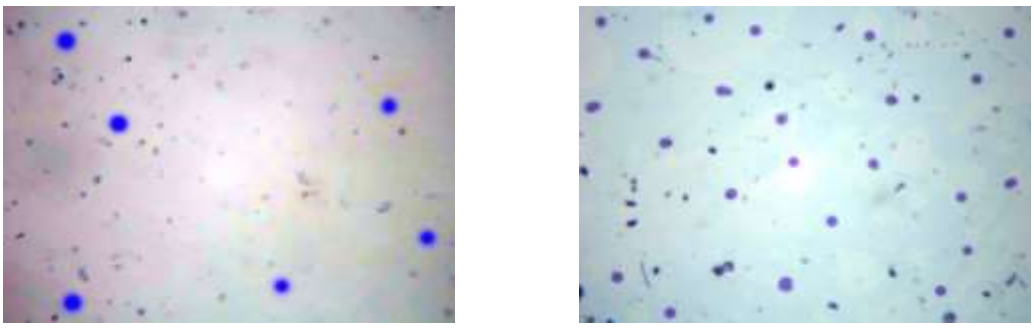
c. Neutrase hydrolyzed β-casein BAPs



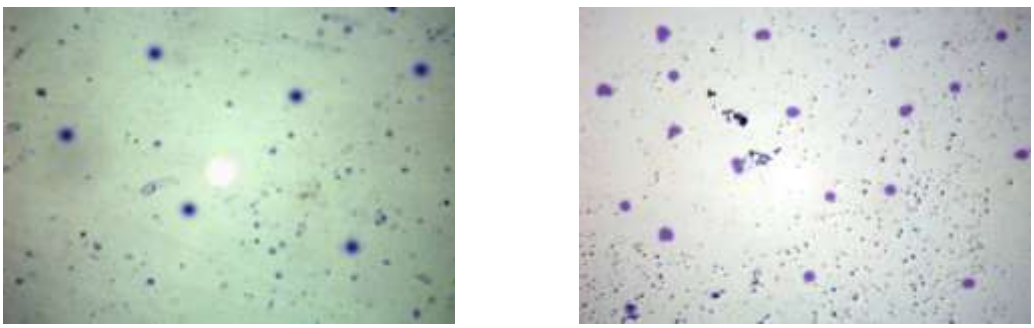
d. Trypsin hydrolyzed β-casein BAPs

Fig 1: Microscopic images of macrophages before and after incorporation of BAPs of casein fractions

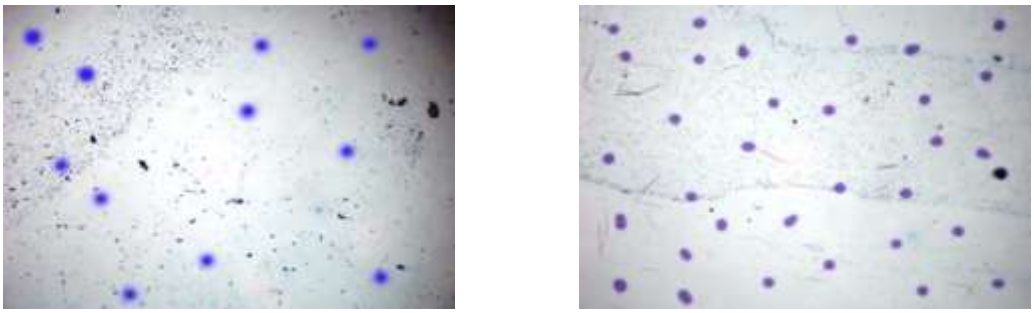
Before After



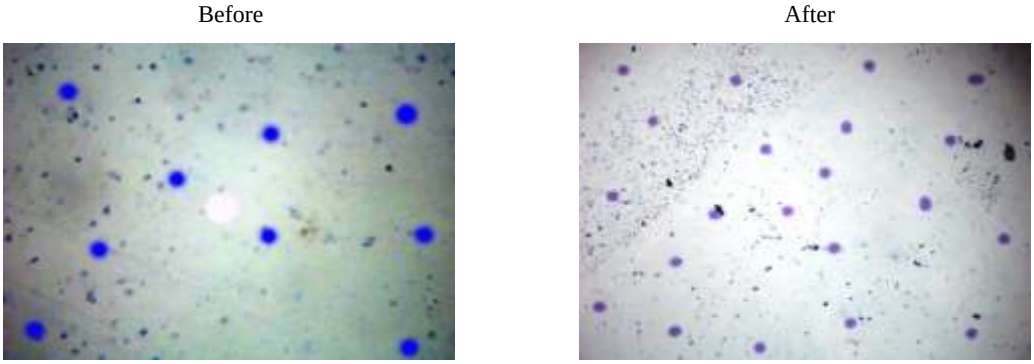
a. Neutrase hydrolyzed α-Lactalbumin BAPs



b. Trypsin hydrolyzed α-Lactalbumin BAPs



c. Neutrase hydrolyzed β-Lactoglobulin BAPs



d. Trypsin hydrolyzed β-Lactoglobulin BAPs

Fig 2: Microscopic images of macrophages before and after incorporation of BAPs of whey proteins

Influence of feeding weaning foods incorporated with neutrase hydrolyzed BAPs of α-casein and β-Lactoglobulin on Total Leucocyte Count (TLcC): Influence of feeding weaning foods to mice incorporated with neutrase hydrolyzed BAPs of α-casein and β-Lactoglobulin at 5 and 10 percent level on total leucocyte count in mice was determined. The total leucocyte count of control (Table-5) was in the range of 3600 to 4100 cells/mm^3 of blood. Weaning food containing BAPs of α-casein (F_1) was in the range of 3,800 to 8,400 cells/mm^3 of blood at 5 percent level and F_2 at 10 percent level (3,900 to 13,500 cells/mm^3 of blood) and for weaning food containing BAPs of β-lactoglobulin (F_3) at 5 percent level showed a range of 3,700 to 8,000 cells/mm^3 of blood and F_4 at 10 percent level the range was 3,850 to 11,700 cells/mm^3 of blood. It is very much clear from the values of the table that, the higher levels of incorporation (10 percent) of BAPs of both α-casein (F_2) and β-lactoglobulin (F_4) in weaning food fed to mice showed higher TLcC when compared to 5 percent level and control. Weaning food incorporated with BAPs of α-casein derived from neutrase hydrolysis showed higher values of TLcC (13,500 cells/mm^3 of blood) than the BAPs of β-lactoglobulin (11,700 cells/mm^3 of blood). The increase in the TLcC values in case of α-casein is due to reason that, it contains the residues (Thr-Thr-Met-Pro-Leu-Trp) in the region of 194-199 which contributes to the hike in level of leucocytes. α-casein and β-lactoglobulin have substantial amounts of glutamylcysteine groups which supply the amino acid precursors (L-glutamate, L-cysteine and Glycine) necessary for the formation of glutathione (L-glutamylcysteinylglycine), which is responsible for immune-enhancing effect. Antigen presenting cells such as, macrophages and B- lymphocytes prefer cysteine for glutathione synthesis which is required to initiate the immune response then feed the lymphocytes, thus, the cysteine acts as an immuno-regulatory signal, which develops a metabolic pathway which gets utilized for enhancing immune properties.

Table 5: Influence of feeding weaning foods with neutrase hydrolyzed BAPs of α-casein and β-Lactoglobulin to mice on Total Leucocyte Count*

Type of weaning food	Feeding regime (Days)			
	0	10	20	30
F_0	3,600	3,800	3,900	4,100
F_1	3,800	6,000	7,200	8,400
F_2	3,900	8,800	11,100	13,500
F_3	3,700	5,200	7,900	8,000
F_4	3,850	7,200	9,400	11,700

Note: All values are average of 6 trials
* Expressed as cells per mm^3 of blood

- F_0: Control group containing weaning food with unhydrolyzed casein
- F_1:Weaning food containing neutrase hydrolyzed α-Casein BAPs incorporated at 5 percent level
- F_2:Weaning food containing neutrase hydrolyzed α-Casein BAPs incorporated at 10 percent level
- F_3:Weaning food containing neutrase hydrolyzed β-Lactoglobulin BAPs incorporated at 5 percent level
- F_4:Weaning food containing neutrase hydrolyzed β-Lactoglobulin BAPs incorporated at 10 percent level

Effect of type of weaning food on gain in body weight of weanling mice: All the laboratory animals showed increased

body weight during the period of feeding (Table- 6). During the entire feeding regime of 30 days the experimental animals fed with F_1, F_2, F_3 and F_4 diets at 10% level showed increased body weight consequently the increased gain in body weight when compared to other groups maintained on 5% level diet. Results of the table revealed that, the gain in body weight for different feeding groups and for different feeding regime were significantly different ($p \leq 0.05$). It is also clear from the values (Table: 5) that, mice fed with 10 percent BAPs incorporated weaning food (F_2 and F_4) resulted in higher weight (27.9 and 27.3 g) than either F_1, F_3 at 5 percent BAPs incorporated weaning food (26.9 and 25.4 g) or control diet (24.9 g).

Table 6: Average body weight* and biological indices of weanling mice fed with different types of weaning foods

Type of weaning food	Feeding regime (Days)				CD ($p \geq 0.05$)
	0	10	20	30	
F_0	13.0	27.5 (14.5)	47.8 (20.3)	72.7 (24.9)	0.63
F_1	13.7	28.6 (14.9)	50.1 (21.5)	77.0 (26.9)	
F_2	14.4	30.1 (15.7)	53.2 (23.1)	81.1 (27.9)	
F_3	14.3	29.6(15.3)	51.8 (22.2)	77.2 (25.4)	
F_4	14.4	29.4 (15.5)	52.8 (22.9)	80.1 (27.3)	
CD ($p \leq 0.05$)	0.54				

Note: * Expressed in grams
All values are average of 6 trials (weight gain)
Values in parenthesis indicate gain in body weight

- F_0: Control group containing weaning food with unhydrolyzed casein
- F_1:Weaning food containing neutrase hydrolyzed α-Casein BAPs incorporated at 5% level
- F_2:Weaning food containing neutrase hydrolyzed α-Casein BAPs incorporated at 10% level
- F_3:Weaning food containing neutrase hydrolyzed β-Lactoglobulin BAPs incorporated at 5% level
- F_4:Weaning food containing neutrase hydrolyzed β-Lactoglobulin BAPs incorporated at 10% level

Conclusion

The present investigation was mainly focused on isolation and characterization of BAPs derived from enzymatic modification of casein and whey proteins using proteolytic enzymes (neutrase and trypsin), their incorporation in weaning foods to enhance immunomodulatory activity. BAPs derived from enzymatic modification of buffalo milk proteins using proteolytic enzymes (neutrase and trypsin). Compositional features of BAPs revealed that, the BAPs obtained from neutrase hydrolysates of α-casein and β-lactoglobulin showed higher levels of nitrogen and protein percentage when compared to BAPs in hydrolysate obtained by trypsin enzyme. BAPs produced from neutrase hydrolyzed α-casein and β-Lactoglobulin showed increased number of macrophages. The BAPs derived from α-casein contains the residues in the region of 194-199 (Thr-Thr-Met-Pro-Leu-Trp) which stimulate the immune system that are responsible for phagocytosis of peritoneal macrophages in mice compared to BAPs of β-lactoglobulin and increased total leucocyte count. Weaning food incorporated with neutrase hydrolyzed α-casein BAPs (F_2) and β-lactoglobulin BAPs (F_4) at 10% level showed increased body weight in mice which is mainly attributed to the better utilization of nutrients especially the available essential amino acids, peptides and increased bio-availability of macro minerals, their easy assimilation and enhanced calcium absorption.

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