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α-glucosidase and α-amylase Inhibitory Properties of A1 and A2 Cow Milk Casein Hydrolysate

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A B S T R A C T

This research intended to examine the *in vitro* health-promoting benefits (namely α-glucosidase, and α-amylase inhibition) of A1 and A2 cow milk casein digested with trypsin. Both A1 and A2 casein hydrolysates manifested α-glucosidase, and α-amylase inhibition, whereas improved activity was recorded with progress in time and degree of hydrolysis. The results indicated that hyperglycaemia was partially control by α-glucosidase, and α-amylase inhibitory properties of casein-derived peptides. It can use as functional food ingredients in the diet of patients with type 2 diabetes.

Introduction

Cow milk serves as a foremost source of nutrition for neonates. Milk proteins release functional bioactive peptides upon enzymatic hydrolysis, using microbial and plant-derived enzymes (Korhonen and Pihlanto, 2003; Fuglsang *et al.*, 2003). Casein (αs1, αs2, β, and κ-casein) and whey proteins (α-lactalbumin, β-lactoglobulin, and lactoferrin) release bioactive peptides (Nielsen *et al.*, 2017). Casein is considered a high biological value protein due to a high level of the essential amino acid and protein digestibility corrected amino acid score (Boye *et al.*, 2012; Schaafsma, 2000). There exist two variants of β-casein, A1, and A2, based on the amino acid position. At 67th position, A1 casein has histidine whereas, A2 consists of proline.

Diabetes is designated as a lack of insulin secretion and insulin resistance (DeFronzo *et al.*, 2009), and type 2 diabetes mellitus (DM) is the most prevalent form of diabetes. It is responsible for oxidative stress, lipid oxidation, high clotting tendency, and the

inflammatory process (Brand-Miller *et al.*, 2007). Type 2 DM, embroiled by several factors, such as insulin resistance, hyperinsulinemia, impaired insulin secretion, reduced insulin-mediated glucose uptake, and utilization (Tiwari *et al.*, 2002). A swift increase of postprandial glucose level in type 2 DM is due to the hydrolysis of starch by pancreatic α-amylase and glucose absorption by α-glucosidase. An efficient approach to control type 2 diabetes is the inhibition of pancreatic α-amylase and intestinal α-glucosidase. Although there are numerous synthetic pancreatic α-amylase and intestinal α-glucosidase inhibitors available commercially for therapeutic use, their effectiveness is limited by harmful side-effects such as abdominal distension, and flatulence. Casein-derived bioactive peptides possess the potential to inhibit pancreatic α-amylase and α-glucosidase (Jan *et al.*, 2016). α-amylase is an enzyme acting on the α-1,4-glycosidic bond of starch and glycogen to yield dextrin, maltose, and glucose. It is an endo-acting enzyme since it attacks inner glycosidic linkage to break down the starch into glucose before absorption. α-amylase inhibition would detain carbohydrate digestion resulting in a decreased rate of glucose absorption (Rabasa-Lhoret *et al.*, 2004). Prolonged exposure to the high glucose level in type 2 DM patients causes the production of free radicals, particularly reactive oxygen species, due to the negotiated antioxidative mechanism of the body.

Therefore, this research designed to investigate the *in vitro* health-promoting benefits namely α-glucosidase, and α-amylase inhibition of A1 and A2 cow milk casein digested with trypsin at different casein concentrations, and at a various time of hydrolysis and also the comparison between the casein variant derived from the A1 and A2 cow milk.

Materials and Methods

Milk

For casein hydrolysate preparation, milk was collected from the Cattle and Buffalo Farm, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, where cow's herd genotyped as A1A1 and A2A2 by the Division of Animal Genetics and Breeding (AGB Division) of the institute.

Enzymatic hydrolysis of A1 and A2 casein

Cow milk casein powder was prepared, according to the method narrated by Salami *et al.*, (2011) in the Division of Livestock Products Technology, ICAR-IVRI, Izatnagar. Cow milk whole casein (1%, 3%, and 5% w/v) solution, was prepared by reconstituting the casein in distilled water. The casein solution was heated in a boiling water bath for 5 min to destroy the microorganisms, if present, which might produce proteolytic enzymes throughout the hydrolysis process. Besides this, it denatures the inherent enzymes in milk, if present, and to denature, the proteins which enhance its sensitivity to proteolytic enzymes. Enzyme/substrate ratio of 1:100 (w/w) was kept constant for trypsin (Thakur *et al.*, 2020). The optimum temperature and pH for enzymatic hydrolysis were 37 ^{o}C and 8.0 (Otte *et al.*, 2007). The hydrolysis did accomplish by incubating the samples at 37 ºC for trypsin in a stirred water bath. Representative samples extracted after a definite time interval i.e. 2 h, 4 h, and 6 h of incubation. Each hydrolyzed samples were instantly warmed to 85 ^{o}C for 15 min in a water bath to inactivate the enzymes left in the hydrolysates. Then the samples were cooled and centrifuged in the refrigerated centrifuge (Hermle, High-Speed Universal Refrigerated Centrifuge) at 10,000 rpm for 20 min, and the supernatant was collected and stored at -20 ^{o}C until further use.

Determination of *in vitro* antidiabetic activity of casein hydrolysates

α-glucosidase inhibition assay

α-glucosidase inhibition assay was carried out by following Apostolidis et al.,(2007) protocol. The α-glucosidase inhibitory activity could be measured *in vitro* by determinating the p-nitrophenol (PNP) after hydrolysis of p-Nitrophenyl-α-D-glucopyranoside (PNPG) by the α-glucosidase enzyme. The α-glucosidase assay was exhibited by adding 50 µl of the sample with 100 µl of 0.1 Mphosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/ml) from *Saccharomyces cerevisiae*. After pre-incubation at 25 ^{o}C for 10 min, 50 µl of 5 mM PNPG solution in 0.1 M phosphate buffer (pH 6.9) was added as substrate. The mixture was further incubated at 25 ^{o}C for 5 min. The reaction was stopped by the addition of 1 ml of 0.1 M Na_2CO_3. Before and after incubation, absorbance (A) readings of samples were recorded at 405 nm by a Microplate reader (Thermo Fisher Scientific) and compared to a control, which had 50 µl of buffer solution in place of the sample. The α-glucosidase inhibitory activity was expressed as % inhibition and calculated as follows:

% α-glucosidase inhibition:

$$\frac{(A_{\text{control 405nm(5min)}} - A_{\text{control 405nm(0min)}}) - (A_{\text{sample 405nm(5min)}} - A_{\text{sample 405nm(0min)}}) \times 100)}{(A_{\text{control 405nm(5min)}} - A_{\text{control 405nm(0min)}})}$$

α-amylase inhibition assay

The α-amylase inhibition assay was performed according to the method described by Kim *et al.*, (2004) and Apostolidis *et al.*, (2007) with some modifications. 100 µl 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α-amylase solution (0.5 mg/ml) was premixed with 100 µl of hydrolysate samples. After pre-incubation at 37 ^{o}C for 7 min, 250 µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added as a substrate in phosphate buffer (pH 6.9). The reaction was performed at 37 ^{o}C for 7 min and terminated by the addition of 200 µl of dinitrosalicylic acid (DNSA) reagent (96 mM DNSA and 5.3 M sodium potassium tartrate in 2.0 M NaOH). The reaction mixture was heated for 15 min at 100 ^{o}C and diluted with 2 ml of distilled water. α-amylase inhibitory activity was determined by measuring absorbance at 540 nm.

% α-amylase inhibition =

$$A_{540}^{control} - A_{540}^{sample} / A_{540}^{control} \times 100$$

Statistical analysis

Data are presented as mean ± standard error (SE) of nine independent experiments. Data were analyzed using the SPSS package (SPSS 20, Version 20, IBM, USA). The difference between the mean was compared using a t-test. Further, Significant differences were determined using Tukey tests ($p < 0.05$). Data obtained from the experiments were pooled. Firstly A1 and A2 samples were compared within the group based on the time of hydrolysis and then based on casein concentration furthermore a comparison was made between A1 and A2 at the same time period of hydrolysis.

Results and Discussion

Determination of *in vitro* antidiabetic activity of casein hydrolysates

α-glucosidase inhibition

α-glucosidase inhibitory activity for 1%, 3%, and 5% casein hydrolysates, ranged from 8.80-17.46%, 11.17-23.28%, and 15.34-29.80%, respectively (Fig. 1). The

comparison of α-glucosidase inhibitory activity within the groups unveiled a significant ($p<0.05$) increase in activity of A1 and A2 casein hydrolysates at 2 h, 4 h, and 6 h of hydrolysis in comparison with whole casein (Table 1).

The differentiation among different casein concentrations showed a significant ($p<0.05$) increase of α-glucosidase inhibition in both A1 and A2 casein hydrolysates at 0 h, 2 h, 4 h, and 6 h of hydrolysis beside the highest activity recorded in 5% followed by 3% and 1% (Fig. 1). α-glucosidase inhibition was directly proportional to the hydrolysis time and casein concentration (Table 1).

In the case of 1% casein, no significant difference ($p>0.05$) was observed between A1 and A2 casein hydrolysates, irrespective of the time of hydrolysis. At 3% and 5% casein level, A2 showed significantly ($p<0.05$) higher α-glucosidase inhibitory activity than A1 casein hydrolysate at 4 h and6 h of hydrolysis (Table 1).

Lacroix *et al.,* (2013) stated that β-lactoglobulin and whey protein hydrolyzed with pepsin showed 33% and 36% of α-glucosidase inhibition, respectively, which was higher than the present findings due to utilization of whey protein. Yogurt, chicken essence, and fish sauce showed inhibitory potential against yeast α-glucosidase (Oki *et al.*, 1999). Further, researchers have reported higher α-glucosidase inhibition in the milk products incorporated with various plant extracts. *Allium sativum* enriched cow (15%) and camel (12%) milk yogurt showed higher α-glucosidase inhibition as compared to plain yogurt (Shori*et al.*, 2011). Apostolidis *et al.*, (2007) recorded 74% α-glucosidase inhibitory activity in cranberry enriched cheese. Phytochemicals had a positive impact on health and could be explored further (Bravo, 1998). Aloe gel enriched curd manifested more prominent α-glucosidase inhibition than plain curd (Ramachandran *et al.*, 2014). Ayyash *et al.,* (2018) reported *Lactobacillus plantarum* fermented bovine milk had higher α-glucosidase inhibitory potential than camel milk.

Table.1 α-glucosidase inhibitory potential of A1 and A2 casein hydrolysates with different casein concentrations at various time periods of hydrolysis (Mean±SE)

α-glucosidase inhibitory potential of A1 and A2 casein hydrolysates with different casein concentrations at various time periods of hydrolysis (Mean±SE)								
Casein concentration	α-glucosidase inhibition (%)							
	Time of hydrolysis							
	0 h		2 h		4 h		6 h	
	A1 casein hydrolysate	A2 casein hydrolysate	A1 casein hydrolysate	A2 casein hydrolysate	A1 casein hydrolysate	A2 casein hydrolysate	A1 casein hydrolysate	A2 casein hydrolysate
1% casein	8.80 ± 0.37^{3d}	9.03 ± 0.38^{3D}	12.52 ± 0.35^{3c}	13.48 ± 0.40^{3C}	15.14 ± 0.27^{3b}	15.94 ± 0.26^{3B}	17.10 ± 0.28^{3a}	17.46 ± 0.33^{3A}
3% casein	11.17 ± 0.30^{2d}	11.51 ± 0.24^{2D}	18.37 ± 0.27^{2c}	18.43 ± 0.26^{2C}	20.32 ± 0.31^{2b}	$21.46\pm0.30^{*2B}$	22.14 ± 0.32^{2a}	$23.28\pm0.37^{*2A}$
5% casein	15.34 ± 0.33^{1d}	15.69 ± 0.29^{1D}	24.10 ± 0.31^{1c}	24.73 ± 0.35^{1C}	25.79 ± 0.28^{1b}	$26.87\pm0.27^{*1B}$	28.56 ± 0.28^{1a}	$29.80\pm0.29^{*1A}$
(n=9); *significant ($p<0.05$) difference between A1 and A2 samples Mean±SE with different superscripts row wise (small alphabets show difference among A1 samples; capital alphabets show difference among A2 samples) and column wise (number show difference among concentration) differ significantly ($p<0.05$)								

Table.2 α-amylase inhibitory potential of A1 and A2 casein hydrolysates with different casein concentrations at various time periods of hydrolysis (Mean±SE)

α-amylase inhibitory potential of A1 and A2 casein hydrolysates with different casein concentrations at various time periods of hydrolysis (Mean±SE)								
Casein concentration	**α-amylase inhibition (%)**							
	Time of hydrolysis							
	0 h		2 h		4 h		6 h	
	A1 casein hydrolysate	A2 casein hydrolysate	A1 casein hydrolysate	A2 casein hydrolysate	A1 casein hydrolysate	A2 casein hydrolysate	A1 casein hydrolysate	A2 casein hydrolysate
1% casein	1.49±0.21 3b	1.67±0.22 3C	2.01±0.15 3b	2.29±0.15 3C	3.65±0.18 3a	3.85±0.18 3B	4.41±0.24 3a	4.65±0.26 3A
3% casein	2.72±0.15 2d	2.87±0.17 2C	4.56±0.19 2c	4.67±0.22 2B	5.51±0.23 2b	6.03±0.17 2A	6.66±0.21 2a	6.70±0.24 2A
5% casein	3.89±0.18 1d	4.47±0.23 1D	6.05±0.33 1c	6.26±0.18 1C	7.64±0.29 1b	8.17±0.36 1B	9.14±0.26 1a	9.44±0.19 1A
(n=9); Mean±SE with different superscripts row wise (small alphabets show difference among A1 samples; capital alphabets show difference among A2 samples) and column wise (number show difference among concentration) differ significantly (p<0.05)								

Fig.1 α-glucosidase inhibition of A1 and A2 casein hydrolysates with different casein concentrations at various time periods of hydrolysis

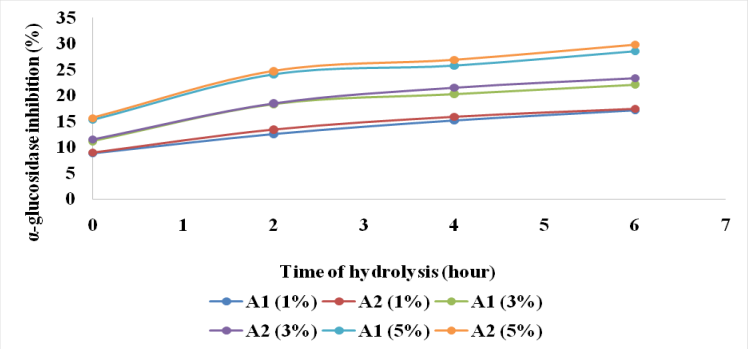
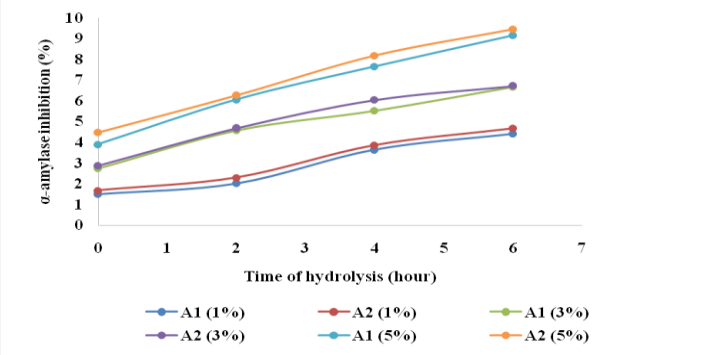


Fig.2 α-amylase inhibition of A1 and A2 casein hydrolysates with different casein concentrations at various time periods of hydrolysis



Pancreatic α-amylase inhibition

The comparison within the groups revealed that, at 1% casein, α-amylase inhibiting activity of A1 casein hydrolysates obtained at 4 h of hydrolysis increased significantly ($p<0.05$) from 0 h and 2 h and hereafter, the increase were not significant ($p>0.05$). However, A2 casein hydrolysate showed a significant ($p<0.05$) increase in activity at 4 h and 6 h. At 3% casein, a significant ($p<0.05$) increase was observed at 2 h, 4 h, and 6 h in A1 casein hydrolysates. However, in the case of A2 casein hydrolysate, a significant ($p<0.05$) increase was observed at 2 h and 4 h of hydrolysis, afterward the increase was non-significant ($p>0.05$). In the case of 5% casein, both A1 and A2 casein hydrolysates showed a significant ($p<0.05$) increase in activity at 2 h, 4 h, and 6 h of hydrolysis (Table 2). α-amylase inhibiting activity of casein hydrolysate varied from 1.49-4.65%, 2.72-6.70%, and 3.89-9.44% for 1%, 3%, and 5% casein respectively (Fig. 2)

The distinction among different concentrations of casein at the same time interval showed a significant ($p<0.05$) increase in α-amylase inhibition with an increase in casein concentration in both the variants of casein hydrolysates (Table 2).

Additionally, no significant difference ($p>0.05$) was observed between A1 and A2 casein hydrolysates at 0 h, 2 h, 4 h, and 6 h of hydrolysis in all casein concentrations (Table 2).

To our knowledge, no records available on the *in vitro* antidiabetic activity of A1 and A2 casein hydrolysate against α-amylase. Jan *et al.,* (2016) reported 44%, 43%, and 41% of α-amylase inhibition in sheep raw milk casein hydrolyzed with trypsin, chymotrypsin, and pepsin, respectively, which was higher than the present finding due to utilization of different species casein. Cow and buffalo milk whey fermented with *Lactobacillus lactis, Lactobacillus delbeurkii* showed 39.18%, 46.59%, and 25.09%, 32.29% α-amylase inhibitory potential, respectively (Vankudre *et al.,* 2015), which was higher than the present result due to fermentation of whey. Ayyash *et al.,* (2017) reported >34% α-amylase inhibition in both bovine and camel milk fermented with different strains of *Lactobacillus* sp. α-amylase inhibitory potential of fermented products could be attributed to the release of bioactive peptides as a consequence of proteolytic enzyme generated by probiotic strains (Da Cruz *et al.,* 2009). Various milk products enriched with numerous plant extracts showed higher α-amylase inhibition, as mentioned. Ramachandran *et al.,* (2014) described a dose-dependent increase in α-amylase inhibition. Sudha *et al.,* (2011) stated that cold water extract and cyclohexane extract of *Aloe vera* exhibited inhibitory potential against porcine pancreatic α-amylase. *Allium sativum* enriched cow and camel milk yogurts showed 38% and 57% α-amylase inhibitory activity than plain cow and camel milk yogurt i.e. 26% and 33%, respectively (Shori*et al.,* 2011), higher than the present study due to the inclusion of *Allium sativum* in yogurt and fermentation of milk. S-allyl cysteine sulfoxide (alliin) and sulfur-containing amino acid in *Allium sativum* manifested α-amylase inhibitory activity (Augusti*et al.,* 1996). Apostolidis *et al.,* (2007) reported 52-100% α-amylase inhibition in cranberry enriched cheese with an increase in dosage from 125µl-500µl. α-amylase inhibitory activity of Roquefort cheese assigned to specific phenolic or secondary metabolite from *Penicillium* sp. (Apostolidis *et al.,* 2007).

In Conclusion, the prevention is as important as the treatment of disease. With the burgeoning interest in nutraceuticals domains,

more and more research is targeted to find the new bioactive compound in different food products. Milk is deemed to be a significant source of bioactive peptides. Additionally, it has a positive impact on the promotion of health and prevention of disease. To mimic digestion A1 and A2 casein was hydrolyzed with trypsin to release functional peptides. Both A1 and A2 casein hydrolysate manifested significantly ($p<0.05$) higher α-glucosidase, and α-amylase inhibitory activity than whole casein. At some points of hydrolysis A2 showed significantly ($p<0.05$) higher activity than A1. The results indicated that hyperglycemia were partially control α-glucosidase, and α-amylase inhibitory properties of casein-derived peptides. Moreover, being derived from a natural protein source, it may have fewer side effects. However, more studies are required to isolate peptides and to use these peptides as medicine.

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Conflicts of interest

There are none potential conflicts between authors and others that bias ourwork.

Abbreviation: Diabetes mellitus (DM), p-nitrophenol (PNP), p-Nitrophenyl-α-D-glucopyranoside (PNPG), Dinitrosalicylic acid (DNSA), h (hour), min (minute).

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