

## SHORT COMMUNICATION

# The effect of milk proteins and curd on serum Angiotensin Converting Enzyme activity and lipid profile in Wistar rats

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**Abstract:** Fermented milk and milk proteins have shown beneficiary health effects in humans. Angiotensin-II, which is formed from angiotensin-I by Angiotensin Converting Enzyme (ACE), plays a major role in regulating the blood pressure in humans and animals. Two experiments were carried out to determine the effects of prolonged consumption of milk proteins (casein & whey) in comparison with fermented milk (curd) on serum ACE activity and lipid profiles in Wistar rats. In experiment I, test groups were given 2 mL of hydrolysed casein or curd, whilst in experiment II, test groups received 2 mL whey and controls received 2 mL of water. The percentage differences obtained for individual animals were analysed after eight weeks. Casein hydrolysate fed group had a 4.3% reduction in serum ACE activity when compared with 3.5% reduction in curd hydrolysate fed group. The mean percentage difference of ACE showed significantly lower values ( $p < 0.05$ ) in both casein and curd fed group when compared with the control. A greater reduction in serum total cholesterol was noted in the curd fed group (7.3%) when compared with the whey fed group (5.4%) and the casein treated group (0.8%). The mean percentage difference was significant ( $p < 0.05$ ) only in the curd and whey treated groups. There was no significant difference in feed intake, body weight, serum high density lipoprotein cholesterol and triglycerides, between the treated groups and the control groups. This indicates that both casein and curd have the effect of lowering serum ACE activity.

**Keywords:** Angiotensin Converting Enzyme activity, casein, fermented milk, lipid profile, whey.

## INTRODUCTION

Milk is a complete nutritional natural food of all mammals and dairy products form a major part of human food. Dairy products and especially fermented milk have been identified as functional foods<sup>1</sup>.

The protein fraction of milk is composed of 80% casein, whey proteins ( $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin) and immunoglobulins<sup>1,2</sup>. Casein ( $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$  and  $\kappa$ )<sup>2,3</sup> forms complexes called micelles with calcium. Milk proteins also contain biologically active peptides. These peptides are formed by the enzymatic hydrolysis of proteins or by the proteolytic activity of the bacteria during microbial fermentation. Microorganisms can form caseino phospho peptides from the protein, casein, and other peptides as well. These peptides survive in the intestine. *In-vitro* studies indicate that milk peptides have an inhibitory effect on Angiotensin Converting Enzyme (ACE) activity<sup>4,5</sup>. Several inhibitors of Angiotensin-I (ANG I) converting enzyme have been isolated from milk protein hydrolysates and this has been validated by *in-vitro* assays<sup>4,5</sup>. Anti hypertensive activity of fermented milk has also been reported and it is believed that specific milk proteins in fermented milk has ACE inhibiting activity<sup>6,7</sup>. Many other peptides also have inhibitory effects on ACE activity<sup>5</sup>.

ACE plays a major role in regulating the blood pressure in humans and animals<sup>7</sup>. This enzyme catalyses the conversion of ANG I to ANG II<sup>8</sup> which is a vasopressor agent that increases the blood pressure by promoting vasoconstriction by directly acting on vascular smooth muscle and increasing the arterial tone by interacting with the sympathetic nervous system both peripherally and centrally<sup>9</sup>. Pharmacological preparations such as  $\beta$ -blockers, calcium-channel blockers, and ACE inhibitors are used to control hypertension<sup>10</sup>. However, these drugs are relatively expensive and have many side effects. If the natural product consumed as food helps in reducing the blood pressure it would greatly benefit mankind.

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It has been reported that the acid producing organisms in fermented dairy products could prolong the life span of the consumer<sup>1</sup>. Fermented foods contain live bacteria that influence the chemical composition of the fermented product, especially milk proteins which result in the availability of amino acids and milk lipids which provide the short chain fatty acids<sup>1</sup>.

Lactose intolerance is a common health problem among Asian adults<sup>1</sup> where 95% of certain Asian people show a more or less advanced low lactase activity<sup>11</sup>. Fermented milk is of high nutritive value and is commonly available as a source of natural protein.

The hydrolysed form of casein and curd used in *in-vitro* studies had a higher inhibiting activity on ACE than fresh curd, milk and whey. This indicates that enzymatic hydrolysis provides smaller peptides that have a stronger ACE inhibiting activity. Several *in-vitro* studies<sup>4-6</sup> and few *in-vivo*<sup>7</sup>, including limited human studies<sup>12</sup> have been performed to validate these effects of the peptides. Hence hydrolysed casein and curd were used in our study.

Very limited work has been reported in *in-vivo* models for ACE inhibiting activity and the lipid profile being measured together. The present study would add new information to the available literature.

An animal study was carried out on male Wistar rats to test the effects of casein hydrolysates, whey or curd (obtained from a local dairy product) hydrolysates, on serum ACE activity and lipid profile.

As caseino phospho peptides (casein hydrolysates) isolated from milk are not commonly consumed by humans, whole curd hydrolysate was used in the animal experiment.

## METHODS AND MATERIALS

A study was carried out using two different experiments to study the effect of casein, whey and curd on male *Wistar* rats. Ethical clearance was obtained from the Ethical Committee, Medical Research Institute, Colombo.

In experiment I, 24 male Wistar rats (8 wks old) were randomly allocated into 3 groups, and in experiment II 14 male Wistar rats (14 wks old) were randomly divided into 2 groups. Fourteen weeks old rats were selected in the 2<sup>nd</sup> experiment to avoid the increase of value with age for the serum parameters assayed. The pre experimental characters of animals such as body weight (BW), ACE activity, total cholesterol (TC), high density lipoprotein

cholesterol (HDL), and triglycerides (TG) did not show any significant differences ( $p > 0.2$ ) between the test and control groups. Each animal in the group was identified by the colour tagging method. They were provided with WHO standard diet, water *ad libitum* and housed in rat cages under standard condition.

In experiment I each rat in the test group was fed with either 2 mL of casein or 2 mL of curd hydrolysates and the animals in the control group received 2 mL of drinking water every morning *via* gastric intubation for a period of 8 wks. In experiment II each rat in the test group was given 2 mL of whey, while the animals in the control group received 2 mL of drinking water daily in the morning *via* gastric intubation for a period of 8 wks.

The oral dose of 2.16 g / 2 mL of casein or curd hydrolysates, which was equivalent to an average intake of 80 g curd by an adult human, was administered *via* gastric intubation. This animal dose was calculated according to a standard drug dose chart conversion table which gives a conversion factor of 0.018 for 200 g body weight of rats. The human consumption of 80 g was given to rats having a mean body weight of 300 g.

The casein sample was prepared by centrifuging the milk at 17,000 g at 4°C for 20 min. The fat in the top layer was removed and was filtered through a muslin cloth and the filtrate was re-centrifuged to ensure the maximal removal of fat<sup>13</sup>. Acetic acid was added to the filtrate to bring the pH slightly below the iso-electric pH 4.6 and the pH was then adjusted to 4.6 using sodium acetate. The weak acid base buffer increases the stability of the precipitate. The casein precipitate was filtered through a muslin cloth and washed several times with water and the precipitate was dried at 45°C in an air flowing oven<sup>14</sup>. The filtrate obtained was centrifuged at 4000 rpm for 10 min to remove any remaining casein and the whey was separated.

The casein sample isolated from milk or a locally manufactured curd sample which was produced using two bacteria species *viz.*, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* as the starter culture was subjected to sequential enzymatic digestion. For the preparation of 100 mL hydrolysates, 108 g casein was dissolved in 25 mL hot water and 108 g curd was dissolved in 25 mL distilled water and they were subjected to sequential enzymatic digestion after cooling. Pepsin was added at pH 1.5 (1 N HCl) and incubated at 37°C for 24 h in a shaking water bath. The pH was adjusted to 7.8 (1 N NaOH) and digested with pancreatin and trypsin for 24h at 37°C. The samples were mixed and the variation in the pH due to the product formed was monitored at time to

time and readjusted to obtain the optimum pH for their activity.

The samples were heated to 80°C for 5 min to inactivate the enzyme activity<sup>5</sup> and pH was adjusted to 7. The samples were diluted to 100 mL to obtain the final concentration of 2.16 g / 2 mL which was used for oral feeding. The hydrolysates were stored at -20°C for a week.

Blood samples were collected from the lateral tail vein into vials without anticoagulant from anaesthetised rats after 12-14 h fasting. The animals were anaesthetised using ether. Samples were collected at the beginning (0 wk) and at 4 and 8 wks during the experiment.

Blood samples were centrifuged after 1/2 h and serum samples were separated and stored at -20°C pending analysis.

The laboratory analysis was performed within 2 d of collection of samples for ACE activity, TC, HDL and TG. The LDL cholesterol concentration was not determined.

The serum ACE activity was measured using some modification in the incubation time and volume to the original Cushman DW and Cheung HS<sup>4,5,7</sup> method in order to increase the efficiency of the assay.

ACE catalyses the hydrolysis of Hippuryl-L-Histidyl-L-Leucine (HHL-sigma product No H-1635) to hippuric acid. HHL buffer (20 µL) was freshly prepared by dissolving in HEPES sodium salt (sigma product No H-7006) HCl buffer and was incubated at 37°C for 5 min. A serum sample of 50 µL or ACE (0.33 units/mL) enzyme solution was added and incubated at 37°C for 15 min. The volume of serum and enzyme concentration was modified in order to improve the efficiency of ACE activity. HCl was added to inhibit the reaction and the supernatant was extracted into 2 mL of ethyl acetate. From the clear upper layer of each vial 1 mL was pipetted into test tubes and was placed in a boiling water bath for 20 min and 3 mL of de-ionized water was added after the ethyl acetate has evaporated. The hippuric acid formed was detected by recording the absorbance at 228 nm in a spectrophotometer.

*Unit definition:* One unit will produce 1.0 µ mole of hippuric acid from HHL per min in 50 mM HEPES and 300 mM NaCl at pH 8.3 at 37°C. Serum was analyzed for TC, HDL cholesterol, and TG using a commercial assay kit method (Accurex Biomedical Pvt. Ltd.).

The TC and TG were assayed using the enzymatic hydrolysis process. The HDL-cholesterol was assayed

using HDL-cholesterol precipitating reagent containing phosphotungstic acid and magnesium chloride that precipitates chylomicrons, LDL and VLDL. HDL cholesterol remains in the supernatant.

Statistical analysis was carried out with Microsoft Excel and Student t-test was performed with SPSS. The p value <0.05 was considered to be significant. The percentage difference was calculated for individual animals for the parameters assayed and it was compared with the control values.

$$\% \text{ differences} = \frac{\text{Value at the end of the experiment} - \text{Initial value}}{\text{Initial value}} \times 100$$

## RESULTS

The mean value of body weight and each blood parameters of animals were similar in all experimental groups except for ACE activity values obtained in curd hydrolysate treated groups in experiment I. The high standard deviation obtained for ACE activity may be due to the biological variation of individual animals (Table 1).

At the end of 8 weeks (Table 2), animals administered with casein had the highest reduction of 4.3% for the ACE activity when compared with the control. Furthermore, all the animals in the casein treated group had a lower ACE value than the initial value. The curd fed group also showed a reduction of 3.5% for the ACE activity. The percentage reduction was significant in both the casein and curd fed group when compared to the control. The control group showed an increase of 0.2% for the ACE activity. This indicates that after eight weeks the intake of casein and curd hydrolysates had significant reduction in ACE activity when compared with that of control.

The serum TC, TG, HDL cholesterol and BW values of animals in all test and control groups increased with their age as the experiment progressed. In the curd fed group the percentage increase in serum TC was lowest and was significantly different (p=0.026) when compared with the control.

The whey fed group (Table 2) had a reduction of 3.2% in the body weight, 0.7% in the ACE activity, 5.4% in the TC and 4.5% reduction in the TG levels respectively. The percentage difference in serum TC showed a significantly lower value (p<0.036) when compared with the control. The other parameters assessed did not show any significant difference when compared with the control.

**Table 1:** Effect of feeding casein and curd hydrolysates on serum ACE activity and lipid profiles of Wistar rats

Experimental groups	Study period	ACE (Units/L)	TC (g/dL)	HDL (g/dL)	TG (g/dL)	BW (g)
Casein†	0 wks	474.8 ± 88.1	79.0 ± 2.1	34.4 ± 4.3	207.8 ± 33.9	192.3 ± 13.7
	4 wks	463.7 ± 86.4	87.6 ± 13.4	Not measured	211.4 ± 33.5	354.4 ± 14.6
	8 wks	455.6 ± 84.8	93.0 ± 9.7	39.4 ± 3.0	228.3 ± 33.8	404.5 ± 29.5
Curd†	0 wks	439.6 ± 108.4	77.62 ± 2.4	33.3 ± 4.3	202.2 ± 38.8	195.0 ± 11.5
	4 wks	432.6 ± 112.6	84.6 ± 2.7	Not measured	207.7 ± 35.8	344.9 ± 14.9
	8 wks	426.1 ± 112.1	87.2 ± 3.1	40.9 ± 1.9	225.3 ± 38.8	400.8 ± 22.5
Control I†	0 wks	476.3 ± 73.6	79.5 ± 12.3	32.0 ± 3.5	208.2 ± 41.8	193.8 ± 7.0
	4 wks	482.3 ± 68.5	89.9 ± 12.4	Not measured	215.3 ± 38.4	348 ± 10.8
	8 wks	487.1 ± 73.0	94.8 ± 13.6	40.6 ± 2.7	232.9 ± 40.0	402.3 ± 16.1
Whey*	0 wks	450.1 ± 98.6	91.0 ± 15.3	41.5 ± 4.0	195.1 ± 14.4	404.3 ± 18.9
	8 wks	450.5 ± 94.3	88.2 ± 12.0	41.6 ± 4.0	186.5 ± 11.7	451.1 ± 26.6
Control II*	0 wks	445.5 ± 76.4	89.7 ± 17.0	45.5 ± 4.1	191.1 ± 24.7	396.9 ± 14.3
	8 wks	449.6 ± 75.6	91.9 ± 16.3	45.6 ± 3.1	191.2 ± 22.3	455.4 ± 20.1

The values given are mean ± standard deviation.

ACE - Angiotensin Converting Enzyme activity, TC -Total cholesterol, HDL - High density lipoprotein cholesterol, TG - Triglycerides, BW - Body weight.

†Age of rats used in the experiment was 8 wks.

\*Age of rats used in the experiment was 14 wks.

**Table 2:** Percentage changes in serum analytes in casein, curd and whey treated groups when compared to the control group after 8 wks of treatment

Experimental groups (I & II)	Percentage change				
	ACE	TC	HDL	TG	BW
Casein	*4.3	0.8	12.0	2.45	-2.96
Curd	*3.5	*7.3	3.7	0.7	1.9
Whey	0.7	*5.4	0.9	4.5	3.2

\*p<0.05 in the Student t-test was considered as significant.

ACE -Angiotensin Converting Enzyme activity, TC - Total cholesterol.

HDL-High density lipoprotein cholesterol, TG- Triglycerides, BW-Body weight.

Experimental animals were male Wistar rats.

## DISCUSSION

The ACE inhibiting activity of casein, whey and the impact of prolonged intake of curd on lipid profile were investigated in the present study.

Similar variations in the standard deviation for the ACE activity have been reported in other animal and human studies as well<sup>15,16</sup>. On a rat model, standard deviation of 98.2 U/L was observed in a control group and 46.5 U/L in the diabetes group<sup>15</sup>, and in a human study a standard deviation of 36.8 U/L was observed where they suggested the variation may be due to the gene polymorphism of ANG I converting enzyme<sup>16</sup>.

Treatment with casein, curd hydrolysates or whey for 4 weeks did not result in a significant change in any of the parameters studied. This shows that the beneficial effects of casein, whey and curd can be seen only after a prolonged (8 weeks) intake.

Previous *in-vitro* studies reported that ACE activity can be inhibited by peptides such as Ile-Tyr, Val-Pro-Pro, Lys-Val-Leu-Pro-Val-Pro<sup>7</sup> and Ile-Pro-Pro<sup>5</sup>. The ACE inhibiting drug captopril is a sulfhydryl containing dipeptide proline<sup>10</sup>. This suggests that peptides present in both the casein and curd hydrolysates may play a role in the reduction of ACE activity of rats fed with casein and curd hydrolysates.

The cholesterol lowering activity seen in curd hydrolysates and whey treated group may be attributed to the lipolytic action of bacteria present in the fermented milk<sup>1</sup>. Thus, the low serum cholesterol observed in the present study may have been due to the production of short chain fatty acids by these bacteria. Short chain fatty acids such as acetic acid, propionic acid, butyric acid<sup>17</sup> have a feedback inhibition on the HMG CoA reductase enzyme which regulates the rate limiting step in the cholesterol synthesis<sup>17, 18</sup>.

The rats were housed under standard cages. This may have restricted their movement which would have been a possible cause for lower HDL contribution in these animals. It has been shown, about 60% of the serum total cholesterol is contributed by the HDL cholesterol and 30% by the LDL cholesterol in rats, in comparison to 30% from HDL and 60% in the form of LDL in humans<sup>19</sup>.

The practitioners of folk medicine in Sri Lanka had been advising their patients with high serum cholesterol, to avoid regular intake of curd and yoghurt. However, this study shows that long term consumption of curd by adults, which is comparable to daily intake, decreases the cholesterol level in rats.

The present study suggests that the long term intake of casein helps to inhibit the ACE activity without any significant differences in the lipid profile, and the long term intake of curd inhibits the ACE activity as well as reduce the serum cholesterol. Further studies with other animal models and human subjects are necessary to recommend the health benefits of milk proteins and curd for man.

Casein and curd hydrolysates have a potential to reduce the ACE activity in Wistar rats. Moderate intake (80 g/day) of curd may favour the reduction of the total serum cholesterol and ACE. This suggests that casein and curd could be of value as an ACE inhibitor, and curd and whey can be recommended in the management of hypercholesterolaemic subjects.

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