In Vivo Anti-diabetic and Biological Activities of Milk Protein and Milk Protein Hydrolysate

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Abstract

The effect of milk protein (MP) and milk protein hydrolysate (MPH) as Anti-diabetic agent were investigated in vivo using six groups of normal and type 2 diabetic rats. The results of this study showed that the treatments of diabetic rats by oral intake of MP or MPH significantly reduced the concentrations of plasma glucose, total lipids of blood plasma, triglycerides, total cholesterol, LDL and VLDL in rat plasma. Also, the treatments of diabetic rats by oral intake of MP or MPH significantly increased the globulin value and HDL, while the concentration of urea, creatinine and bilirubin were reduced. In addition, oral intake of MPH has no affective on acid phosphatase (ACP), alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST) activities in blood plasma and liver of normal rats and protective its concentrations in diabetic rats. The present results concluded that MP and MPH could be used as anti-diabetic agents.

Keywords: Milk protein; Protein hydrolysate; Bioactive peptides; Anti-diabetic peptides

Introduction

Type 2 diabetes mellitus is a public health crisis that affects the economies of the world, especially developing countries. The worldwide incidence of type 2 diabetes is increasing. It was estimated in 2000 that there were 171 million diabetics. This will reach to 366 million people in 2030 [1]. In 2003 the expert committee of the American diabetes association proposed to eliminate the terms insulin and non-insulin-dependent diabetes mellitus and their acronyms (IDDM and NIDDM) [2]. Type 1 diabetes is actually known as IDDM [3]. Type 2 diabetes is a heterogeneous clinical syndrome characterized by elevated blood glucose levels due to defective insulin secretion and/or insulin action, so this type known as NIDDM [4]. When insulin secretion cannot compensate for insulin resistance, type 2 diabetes develops [5].

Milk proteins are considered to be the most important sources of bioactive peptides. The beneficial health effects of milk bioactive peptides are classified as cytoprotective, mineral binding, antimicrobial, immunomodulatory, blood-pressure lowering (Angiotensin-converting enzyme, ACE-inhibitory), antithrombotic, antioxidant and opioid like, in addition to cholesterol-lowering [6]. Bioactive peptides released from milk proteins in the gastrointestinal tract by the action of digestive enzymes such as pepsin, trypsin, chymotrypsin, carboxy and aminopeptidases. The physiological activity of bioactive peptides depends on their ability to maintain integral state during transport to the various functional systems of the body [7]. Studies suggested that the antidiabetic properties of milk protein are primarily attributable to its content of bioactive peptides which, could arouse the secretion of gut-derived hormones and/or inhibit enzymes involved in glucose homeostasis.

β-Casomorphin-7 (β-CM-7) was described as the most representative milk-derived bioactive peptide. It was found that after oral intake of β-CM-7 to diabetic rats, the level of plasma insulin increased, and reduced the elevated in plasma glucagon level [8].

On the other side, the inhibition of dipeptidyl peptidase IV (DPP-IV) has been proposed as a new avenue for the treatment of Type 2 diabetes (T2D). It hydrolyses incretin hormones such as glucose dependent insulinoïtopetic (GIP) and glucagon-like polypeptide-1 (GLP-1) [9]. Those incretins can enhance insulin secretion from pancreatic beta cells in the presence of nutrients in vivo. Milk protein is the natural source of DPP-IV inhibitors. Nangioniema et al., [10] found that three amino acids released from whey proteins hydrolysate (Met, Leu and Trp) and eight dipeptides (Phe-Leu, Trp-Val, His-Leu, Glu-Lys, Ala-Leu, Val-Ala, Ser-Leu and Gly-Leu) inhibited DPP-IV. On the other hand, Trp and Trp-Val inhibited xanthine oxidase (XO) and DPP-IV. Trp was a non-competitive inhibitor of XO and a competitive inhibitor of DPP-IV [10]. The present study aimed to investigate the biological effects of milk protein (MP) and milk protein hydrolysate (MPH) as anti-diabetic agents in alloxan-induced diabetic rats.

Materials and Methods

Materials

Cow milk protein concentrate (about 70% protein) (MP) was obtained from Fonterra Ltd, Auckland, New Zealand. Other chemicals were obtained as follows: Trypsin 2000 U/g (EX pancrease) from Loba Chemie, India. Alloxan was obtained from Alpha Co. India; it was dissolved in saline solution (0.9% sodium chloride, pH 7). And the dose of alloxan (120 mg/kg BW) was chosen to induce diabetes in rats according to previous studies [11]. Acetonitrile, HPLC grade from Scharlau, Spain. Trifluoroacetic acid HPLC grade SdS, France.

Animals

Sixty male healthy albino rats with weights ranging from 190 to 210 g were obtained from Institute of Graduate Studies and Research (IGSR), Alexandria University, Egypt.

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Methods

Preparation of milk protein hydrolysates: Milk protein hydrolysate (MPH) was prepared based on the method described by Otte et al., [12]. Protein solutions (2% w/w on protein basis) were made by dispersion of approximately 2.85 g of milk protein concentrate (according to the protein content) in 97.15 ml of distilled water to final weight 100 g, and stirred for 1 h at room temperature. Solutions were hydrated overnight at 5°C. The pH (~ 6.9) was adjusted to 7.5 with 1% NaOH in half of the samples. Trypsin enzyme solution (80 mg ml⁻¹) was made in distilled water immediately prior to use. For each hydrolysis experiment, 100 ml of protein solution was pre-incubated at 40°C for 10 min. After withdrawal of 1 ml sample (zero time sample), the hydrolysis process was started by addition of 0.5, 1.0, 1.5 and 2.0 ml of trypsin solution to the remaining 100 ml of protein solution, and vortex mixing for 30 s. Enzyme (Units) to substrate ratio were in range of 80:100, 160:100, 320:100 (2X, 4X, and 8X) respectively. The reaction mixture was incubated at 40°C, and intervals samples (1 ml each) were withdrawn after 3, 6, 9, 20 and 24 h of hydrolysis. Enzyme was inactivated by heating at 90°C for 15 min, and then cooled for 20 min in ice bath and then centrifuged (Sigma centrifuge 113 VWR International, Germany) for 10 min at 10,000 xg, the supernatant was used for further analyses as described below.

Analysis of peptides by reverse-phase high-performance liquid chromatography (RP-HPLC): Separation of the peptide extracts was carried out using a HPLC system (Agilent Technologies 1260) according to the method described by Awad et al., [13]. Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. WP 300 RP-18, 5 μm, 250 x 4.6 mm from Merck was used for analyses. Samples were filtered through a 0.2 μm membrane filter (Millipore Corp., Bedford, MA). 50 μl of peptides extract were injected into the column. The sample was eluted with a four-steps linear gradient over a period of 75 min, starting with solvent A for 5 min, then linear increase from 0 to 50% solvent B at a flow rate of 1.0 ml/min for 60 min, followed by 50% solvent B for 5 min and by a linear gradient from 50 to 100% B developed over 5 min to clean the column. Before the next injection, the column was allowed to equilibrate with solvent A for 15 min. Separation was conducted at 30°C and peptides monitored at 200 and 230 nm.

Animals and treatments: The animals were housed in cages (5 per cage) under proper environmental conditions. Rats were fed commercial pellets. The chemical analysis of the pellets showed that it contained 17.5% crude protein, 14.0% crude fiber, 2.7% crude fat and 2200 Kcal/kg [14]. Tap water was provided ad libitum and kept for two weeks for acclimatization and maintained at 25 ± 1°C with 12 h dark and light cycle [15]. According to the results of RP-HPLC, sample of treatment trypsin 8X at pH 7.5 and 40°C for 20 h was selected to be used in animal treatments (Figure 1). The experimental period was six weeks after stabilization of diabetes for one week and the animals were divided into six major groups as following:

Blood biochemical parameters and enzyme activities: The blood samples were collected in tubes with heparin (anti-coagulant). The heparinized blood samples were placed immediately on ice. Plasma samples were obtained by centrifugation at 860 xg for 20 min, and were stored at -80°C until used for analyses. Stored plasma samples were analyzed for total protein (TP) by the Biuret method [16]. Albumin (A) concentration was determined [17]. Plasma glucose, urea and creatinine concentrations were measured as previously described [17-19] respectively. Plasma total bilirubin was determined as previously described [20]. Plasma concentrations of total lipids (TL), cholesterol and triglycerides (TG) were as previously described [21-23], respectively. High-density lipoprotein cholesterol (HDL-C) was determined [24]. Low-density lipoprotein cholesterol (LDL-C) was determined by the calculation (cholesterol-(TG/5+HDL)). Very low-density lipoprotein cholesterol (VLDL-C) was calculated by dividing the values of TG by

![Image of RP-HPLC pattern](image-url)
factor of 5. The activities of plasma aspartate transaminase (AST) and alanine transaminase (ALT) were assayed as previously described [25]. Acid phosphatase (ACP) and alkaline phosphatase (AlP) activities were determined in plasma as previously described [26,27], respectively.

Liver biochemical parameters and enzyme activities: Liver was immediately removed at the end of the experiment; weighed and washed using chilled saline solution. Tissues were minced and homogenized (10% w/v) in ice-cold sucrose 10% in a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 10,000 µg for 20 min at 4°C. The resultant supernatant of the organs was used for different enzyme activities according to the previous methods. Concentrations of hepatic AST, ALT, ACP, and AlP were determined.

Statistical analysis: Data were analyzed as a completely randomized design using the general linear model procedure of SAS [28,29]. Means were statistically compared using least significant difference (LSD) test at 0.05 significant levels.

Results

Reverse phase high performance liquid chromatography

The hydrolysate of all MP treatments were eluted by RP-HPLC, chromatograms showed that the trypsin (2X, 4X and 8X) produced more peptides after 24 h than after 20 h of incubation, and the hydrolysate at pH 7.5 produced small peptides than at pH 6.9. According to the evaluation RP-HPLC, the treatment of trypsin 8X at pH 7.5 and 40°C for 20 h was selected to be used in the experiment (Figure 1).

Plasma glucose level of diabetic and non-diabetic rats

Table 1 represents plasma glucose concentration (mg/dl) of male diabetic and non-diabetic rats. Treatment animals with alloxan alone caused a three-fold increase in plasma glucose concentration when compared to control group. Meanwhile, treatment with oral intake MP and MPH of diabetic rats was associated with plasma glucose concentrations significantly lower than those observed with control diabetic rats and higher than those observed with control non-diabetic rats. Glucose concentrations in the MCPHD group were significantly ($p<0.05$) lower than in the MCPPD group. These results showed that the MPH had a greater effect in reduction blood glucose concentration of diabetic rats while there was no effect on normal healthy rats.

Plasma protein profile of diabetic and non-diabetic rats

Table 1 shows the changes of plasma protein concentrations when compared to control groups. The concentration of total protein and globulin concentration of diabetic rats by oral intake of MP and MPH returned the concentrations of the previous parameters when compared to control group. On the other hand, treatment with oral intake of MP and MPH of diabetic rats was returned the concentrations of total protein and globulin to the normal range of control group. The treatment of normal healthy rats by oral intake of MP and MPH had no significant ($p>0.05$) affect found in concentrations of the previous parameters when compared to control group.

Plasma lipid profile of diabetic and non-diabetic rats

Table 2 shows the changes of lipid profile in plasma due to treatment with oral intake of MP and MPH. Treatment with alloxan alone caused a significant increase ($p<0.05$) in plasma total lipid (TL), triglycerides (TG), cholesterol, low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C), while high-density lipoprotein cholesterol (HDL-C) decreased compared to that in control group. However, treatment of diabetic rats by oral intake of MP and MPH returned the concentrations of the previous parameters to near control group concentrations, and the high reduction of total lipid, cholesterol and LDL was found in MPH treatments. Meanwhile, no significant ($p>0.05$) changes were found in the concentration of total lipids, triglycerides, LDL-C and VLDL-C of normal healthy rats when compared with control group. On the other hand, treatment by MPH caused significant ($p<0.05$) increase in HDL-C than control and MP groups. The normal rats treated by MP was significant ($p<0.05$) increase in cholesterol concentration.
compared to control and MPH groups. Also, LDL-C concentration was significant (p<0.05) increase in MP group than MPH group.

**Plasma urea, creatinine and total bilirubin of diabetic and non-diabetic rats**

Treatment with alloxan alone caused a significant increased (p<0.05) in plasma urea (mg/dl), creatinine (mg/dl) and total bilirubin (mg/dl) of diabetic rats (Table 3).

However, treatment with oral intake of MP and MPH of diabetic rats alleviated the effect of alloxan on plasma urea and total bilirubin. The creatinine concentration is decreased in all treated groups except in MPD group when compared to diabetic control group.

There is no significant (p>0.05) differences were found in urea and total bilirubin concentrations between MPH and normal control group. On the other hand, there was no significant (p>0.05) difference in plasma urea and total bilirubin of non-diabetic rats treatment with MP compared to control group, but creatinine was significant (p<0.05) increased. Meanwhile, non-diabetic rats treated by MPH were significant (p<0.05) decreasing in plasma creatinine and total bilirubin, while, there was no significant (p>0.05) difference in urea concentration in compared to control group.

**Liver enzymes activity in plasma and liver extract of diabetic and non-diabetic rats**

Treatment with alloxan alone significantly (p<0.05) increased plasma AST, ALT, ALP and ACP activities and decreased liver tissue AST, ALT, ALP and ACP activities and decreased liver tissue (mg/dl) of diabetic rats (Table 4).

The results showed that there were no significant (p>0.05) difference in plasma AST and ACP of normal rats treated by MP and MPH in compared to normal control group. In addition, there were no significant (p>0.05) difference in plasma ALP activity between MP and normal control groups, while the ALP activity was decreased with MPH compared to control group. Also, ALT activity was decreased with MPH group and not affected in MPH group in compared with normal control group. Concerning the effect of oral intake of MP and MPH on ACP, ALT, ALP and AST activities in liver tissue of diabetic rats (Table 4), the results showed that there was a significant (p<0.05) increased in ACP in diabetic rats treated by MP and MPH in compared with diabetic control. ALP activity was significant (p<0.05) increased in diabetic rats treated by MPH compared with diabetic control and no significant (p>0.05) difference among MPD and MPH D groups. The results cleared that there is no significant (p>0.05) difference in AST Activity between diabetic rats treated by MP or MPH and diabetic control group. Concerning to the effect of oral intake of MP and MPH on ACP, ALP, ALT and AST activity in liver tissue of non- diabetic rats, the results in Table 5 showed that there were no significant (p>0.05) difference in ACP, AST and ALT Activity of normal rats treated by MP and MPH when compared to control group. On the other hand, the ALP activity was significant (p<0.05) decreased after treated by MPH comparing with control, while there were no significant (p>0.05) difference between MP and MPH groups.

**Discussion**

The plasma glucose concentration was increased in diabetes rats [30]. Moore et al., showed that the increase in serum glucose level of diabetic rats indicates the death of pancreatic β-cells of tested animals [31]. Alloxan treatment caused a significant increasing of blood glucose level (p<0.01) when compared to the control group [32]. Bioactive peptides from milk proteins has been linked with serum glucose regulatory properties in humans [33]. The suggested mechanisms include an insulinotropic activity, incretin secretagogue action, as well as activity on different metabolic enzymes involved in the regulation of serum glucose such as dipeptidyl peptidase IV (DPP-IV), a-amylase and α-glucosidase [34]. The free amino acids can directly effect on the β-cell to release insulin [35]. Various studies have highlighted that the insulinotropic activity of whey protein hydrolysate has been related to intestinal amino acid absorption and the increased plasma concentration of free amino acids (Leu, Ile, Phe, Tyr, Thr, Val, Ala

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### Table 3: Effect of oral intake of milk protein concentrate (MPC) and milk protein concentrate hydrolysate (MPCH) on urea, creatinine, and bilirubin levels of male diabetic (D) and non-diabetic rats.

| Parameters | Control | Control D | MPC | MPC D | MPCH | MPCH D |
|------------|---------|-----------|-----|-------|------|--------|
| Urea (mg/dl) | 47.84 ± 0.75<sup>cd</sup> | 79.40 ± 6.88<sup>b</sup> | 46.86 ± 0.96<sup>abc</sup> | 55.25 ± 2.11<sup>c</sup> | 42.0 ± 1.59<sup>bc</sup> | 52.46 ± 0.59<sup>c</sup> |
| Creatinine (mg/dl) | 1.129 ± 0.033<sup>b</sup> | 1.393 ± 0.085<sup>b</sup> | 1.279 ± 0.038<sup>bcd</sup> | 1.339 ± 0.049<sup>b</sup> | 1.012 ± 0.066<sup>bc</sup> | 1.103 ± 0.018<sup>b</sup> |
| Bilirubin (mg/dl) | 2.581 ± 0.047<sup>a</sup> | 3.149 ± 0.180<sup>b</sup> | 2.419 ± 0.109<sup>cd</sup> | 2.490 ± 0.045<sup>bc</sup> | 2.225 ± 0.109<sup>cd</sup> | 2.396 ± 0.050<sup>cd</sup> |

Values are expressed as means ± SE; n = 7 for each treatment group. Mean values within a row not sharing a common superscript letters (a, b, c, d) were significantly different, p<0.05.

### Table 4: Effect of oral intake of milk protein concentrate (MPC) and milk protein concentrate hydrolysate on acid phosphatase, alkaline phosphatase, aspartate amino-transferase (AST) and alanine amino-transferase (ALT) levels in plasma of male diabetic (D) and non-diabetic rats.

| Parameters | Control | Control D | MPC | MPC D | MPCH | MPCH D |
|------------|---------|-----------|-----|-------|------|--------|
| Acid phosphatase (U/L) | 25.39 ± 1.03<sup>b</sup> | 29.52 ± 1.87<sup>a</sup> | 22.34 ± 1.09<sup>b</sup> | 25.76 ± 2.20<sup>ab</sup> | 23.03 ± 1.59<sup>ab</sup> | 26.20 ± 1.84<sup>cd</sup> |
| Alkaline phosphatase (U/L) | 129.6 ± 3.66<sup>b</sup> | 133.2 ± 3.87<sup>a</sup> | 114.9 ± 5.60<sup>bc</sup> | 129.3 ± 8.03<sup>b</sup> | 113.5 ± 6.69<sup>a</sup> | 128.2 ± 8.19<sup>abc</sup> |
| Alanine amino-transferase (ALT) | 27.83 ± 0.62<sup>cd</sup> | 33.51 ± 0.17<sup>b</sup> | 30.47 ± 0.63<sup>c</sup> | 32.04 ± 0.64<sup>b</sup> | 28.77 ± 1.02<sup>b</sup> | 31.03 ± 0.77<sup>a</sup> |
| Aspartate amino-transferase (AST) | 44.63 ± 0.8<sup>cd</sup> | 49.10 ± 1.20<sup>b</sup> | 43.45 ± 0.28<sup>d</sup> | 45.69 ± 0.52<sup>cd</sup> | 44.07 ± 0.42<sup>cd</sup> | 46.02 ± 0.89<sup>a</sup> |

Values are expressed as means ± SE; n = 7 for each treatment group. Mean values within a row not sharing a common superscript letters (a, b, c, d) were significantly different, p<0.05.
The hydrolysates obtained from either whey or casein protein elicited about 50% more gastric secretion than whole protein solutions which was accompanied by increased GIP in plasma during the first 20 min of gastric emptying [38]. Whey-derived bioactive compound is the tripeptide Ile-Pro-Ala, released from β-lactoglobulin hydrolysis, which may act as inhibitor of dipeptidyl peptidase-4 (DPPIV), reducing glucose levels and stimulate insulin [39]. Also, DPP-IV inhibitory peptide Leu-Pro-Glu-Arg-Ile-Pro-Pro-Leu from Gouda type cheese induced a significant reduction of blood glucose in rats [24].

The significant decrease (p<0.01) in total protein, albumin and albumin/globulin (A/G) ratio in diabetic group when compared to the control group [40]. The increase in lipid profiles and decrease in HDL-C were found in diabetic rats [41]. The concentration of TC, TG, cholesterol, LDL-C and VLDL-C were significantly increased in the plasma of alloxan induced diabetic rats compared to control group. While, HDL-C concentration was significantly decreased. Moreover, the high concentration of low-density lipoprotein cholesterol (LDL-c), hypertriglyceridemia (TG) and high-density lipoprotein HDL are established as independent risk factors for diabetes [42].

Increase availability of glucose plasma concentration caused higher increase in VLDL in diabetes mellitus due to for VLDL synthesis and decrease in lipoprotein lipase activity leading to decrease of VLDL from peripheral circulation. In addition, an increased percent body fat was identified with higher concentrations of TC: HDL-C and decreased HDL-C due to decrease in hepatic lipase activity resulting in decrease VLDL clearances which are metabolic abnormalities characterizing metabolic syndrome [43].

However, the feeding with whey protein elevation of blood serum cholesterol in rabbit. This effect was confirmed when a trypptic hydrolysate of β-lactoglobulin (β-Lg) was administered to rats fed a diet rich in cholesterol [44]. On other side the serum urea and creatinine concentrations were significant higher in type II diabetic patients (test subjects) compared to non-diabetic control subjects [45].

The increase of plasma AST, ALT activities, and the decrease in liver of diabetic untreated rats are in agreement other studies [11,46]. The increase in activities of plasma AST, ALT, LDH, AIP and ACP indicated that diabetes may be induced hepatic dysfunction. Therefore, the increase of the activities of AST, ALT, LDH, AIP and ACP in plasma may be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream which gives an indication on the hepatotoxic effect of alloxan [47]. The reduction in liver enzyme activities ALT, AST, ACP and liver of diabetic rats are mainly due to leakage of these enzymes into the blood stream as a result of alloxan toxicity which leads to the liver damage [46]. The elevation of serum AST and ALT activity in the present work may be attributed to the excessive release of such enzymes from the damaged liver cells into the blood circulation. Where, there is an inverse relationship between the liver activity and the level of enzymes in serum, and may be consistent with their greater need for gluconeogenesis substrates or may reflect damage of the hepatic cells due to hepatotoxic effect of alloxan [32]. Other study showed that the whey protein reduced the ALT level of the sera of the patients infected with hepatitis B virus [48].

Conclusion

The obtained results suggested that treatments of diabetic rats by oral intake of MP or MPH significantly reduced the concentration of plasma glucose, total lipid, Triglycerides, total cholesterol, LDL and VLDL. Also, MPH reduced the concentrations of urea, creatinine and bilirubin. Interesting, the treatments of diabetic rats by oral intake of MP or MPH significantly increased the globulin concentration and HDL. The results recommended that milk protein especially milk protein hydrolysate could be a good source of anti-diabetic agents.

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