Genetic engineering of *Lactococcus lactis* to produce an amylase inhibitor for development of an anti-diabetes biodrug

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SUMMARY

Diabetes is known as a multifactorial disease. The treatment of diabetes is complicated due to its inherent pathophysiological factors related to the disease. One of the complications of diabetes is postprandial hyperglycemia. Glucosidase inhibitors, particularly α-amylase inhibitors can help manage postprandial hyperglycemia. The low molecular weight inhibitor of α-amylases called PAMI (peptide amylase inhibitor) inhibits the α-amylase. In this study we cloned this amylase blocker PAMI in *Lactococcus lactis*. Using this *Lactococcus lactis* expressing the PAMI, we prepared yogurt and fed it to diabetic mice models. There was decrease in the blood glucose level after 20 days of oral administration of the yogurt. This product be used as a biodrug in maintaining the blood glucose level in diabetic patients.

KEY WORDS: Lactococcus lactis, Post prandial hyperglycemia, α-amylase inhibitors

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INTRODUCTION

Diabetes mellitus (DM) is a major global health problem affecting more than 185 million people around the world (Amos *et al.*, 1997; Zimmet *et al.*, 1999; Zimmet *et al.*, 2001). The prevalence of type 2 diabetes has increased rapidly worldwide over the past several decades, a phenomenon that has been ascribed to the collision between an inherited predisposition and a westernized environment (Josée *et al.*, 2010). DM can be divided primarily into two types: Type 1, or insulin-dependent diabetes mellitus (IDDM), and type 2, or non-insulin dependent diabetes mellitus (NIDDM) (Kumar *et al.*, 2005).

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The treatment of type 2 diabetes is complicated by several factors inherent to the disease and elevated postprandial hyperglycemia (PPHG) is one of the risk factors (Gin *et al.,* 2000). Glucosidase inhibitors such as α-amylase inhibitors play a major role in managing PPHG in diabetic patients. α-amylases (α-1,4-glucan-4-glucanohydrolases) are a group of glycoside hydrolases widely distributed in microorganisms, plants and animal tissues. They catalyze the hydrolysis of the α-(1,4) glycosidic linkage found in starch components and other related polysaccharides. Amylase inhibitors have been used in the suppression of postprandial glucose levels in diabetic patients (Breuer *et al.,* 2003) and in suppression of the development of insect pests through the impairment of their amylolytic digestion (Octávio *et al.,* 2002). Thus, amylase inhibitors inhibit the action of α-amylase enzyme leading to a reduction in starch hydrolysis which shows beneficial effects on glycemic index control in diabetic patients (Notkins *et al.,* 2002). Proteinaceous α-amylase inhibitors are strong ligands, their binding is generally established through an extended set of intermolecular con-

tacts with several segments of the polypeptide chain involved in the binding. Recently a peptide amylase inhibitor (PAMI) 'GHWYYRCW' has been designed which is reported to be the first low-molecular weight inhibitory motif for α-amylase designed through the use of combinatorial chemistry (Dole ková-Marešová *et al.,* 2005).

Biodrugs are much more complex than chemically synthesized drugs because of their structural heterogeneity and interactions within a given biologic system (Sahoo *et al.,* 2009). The biodrug concept comprises living microorganisms carrying out either bioconversion or biosynthesis in the digestive environment. The bioactive products resulting from bioconversion or biosynthesis can be secreted in the digestive medium, be bound to the cells or accumulate inside the cells and be released in the digestive medium by cell lysis (Blanquet *et al.,* 2001). We used this approach in drug production directly in the digestive environment by ingesting living recombinant *Lactococcus lactis* secreting the amylase inhibitor. *L. lactis* is widely used in the food industry for the production and preservation of fermented products. Their traditional use in the food industry confirms their lack of pathogenicity; they are considered to be generally regarded as safe (GRAS) organisms. *Lactococcus lacti*s is the model lactic acid bacteria (LAB); many genetic tools have been developed and its complete genome has been sequenced (Alexander *et al.,* 2001). Protein secretion by this GRAS bacterium would allow production directly in food product and thus an interaction between the secreted protein and the environment. *L. Lactis* considered is a good candidate because relatively few proteins secreted and this secreted proteins are prone most of the time to extracellular degradation, even in multi-deficient protease strains (Wu *et al.,* 1991). So far there have been plenty of heterologous proteins produced in *Lactococcus lactis* like bacterial antigens, eukaryotic antigens, viral antigens, interleukins, allergens, virulence factors, bacteriocins, reporter proteins and enzymes.

The goal of this study is to use genetically engineered *L. lactis* as a biodrug, which can be used as a delivery vehicle to the gastrointestinal tract delivering the shortest peptide blocker '"GHWYYRCW" and ingest the yogurt made by this *L.lactis* to the streptozotocin-induced insulitis mouse and high fat induced diabetic mouse as a type 1 and type 2 diabetes model respectively. To our knowledge this study has been done for the first time to use *L.lactis* as a therapy against the diabetes.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

The bacterial strains used in this study are *Lactococcus lactis* subsp. *lactis* IL1403 and *Lactococcus lactis* subsp. *cremoris* MG1363, which was kindly provided by Isabelle Poquet (INRA, France) and Søren M. Madsen (Bioneer, Denmark). The plasmid pVE5523 was provided by Jean-Christophe Piard (INRA, France). This 7.8 Kbp vector consist of ampicillin and erythromycin resistant gene with complete constitutive gene expression system facilitated for the extracellular protein secretion, further details can be found Dieye *et al.* (2001). Plasmid construction and manipulation performed in *Escherichia coli* DH5 . *E.coli* cells were grown in Luria broth (Sambrook *et al.,* 1989) at 37°C with shaking. *L. lactis* was grown in GM17 medium (Terzaghi *et al.,* 1975) (Difco, BD, USA) at 30°C degrees without shaking. Where appropriate, antibiotics were added as follows: for *E. coli* and *L. lactis*, erythromycin (75 µg/ml) and ampicillin (75µg/ml).

DNA manipulation and transformation procedures

General molecular biology techniques were performed essentially as described previously (Sambrook *et al.,* 1989). Plasmid DNA was extracted using plasmid purification mini procedure kit (Nucleogen biotechnology, S.Korea). Lysozyme (10 mg/ml) was added prior to lysis step in *L.lactis* and incubated for 30 minutes (37ºC) to prepare protoplasts. Plasmids were established by electroporation as described for *L.lactis* (Langella., 1993).

Cloning of synthetic gene in *L. lactis* and its expression

The synthetic gene encoding PAMI (peptide blocker: GHWYYRCW) was synthesized from cosmo-genetech, S. Korea. (5'-GTGATTGTCGACGGTCACTGGTACTACCGTTGCTGGGATATCATGTAT-3') with *EcoRV* and *Sal1* restriction sites. The plasmid pVE5523 and synthetic gene was digested with the *EcoRV* and *SalI*. These frag-

ments were cloned using double digestion method (Damak *et al.,* 1993). Ligation was confirmed by performing PCR (PTC-100, MJ research. Inc. USA) using primers fp:CAAGCTCGAAATTAACCCTCAC and rp: TTCTCTGTCGCTATCTGTTGC, thus the size of the PCR product was around 800bp. DNA sequencing was performed from cosmo-genetech, S. Korea.

Immunoblotting

We used GHWYYRCW peptide as the positive control and the antibodies synthesized against PAMI in rabbits, both commercially synthesized from cosmo-genetech, S. Korea. The recombinant cloned bacterial samples and the positive control peptide was first blotted on the nitrocellulose paper (Joanne *et al.*, 2002) and then probed with the primary antibody against the peptide and then with the goat anti-rabbit IgG conjugated to horseradish peroxidase (Promega, USA). The peptide was detected upon development of appropriate dot after reaction with the substrate peroxide buffer and enhancer solution (Immunstar WesternC kit, Biorad, USA). The peptide released was quantified by scanning western blot and comparing signals to those of known amounts of PAMI synthesized commercially using chemi-doc (bio-rad, USA). Amounts are presented as milligrams per milliliter of culture corresponded to an OD_{600} of 1.

Animals

Ten-week-old male C57BL/6J mice were purchased from (Central Lab. animal Inc. Japan). The animals were kept maintained in a temperature-controlled room (22ºC) on a 12-h light dark cycle. The study was approved by Chonbuk National University Institutional animal care and use committee, S. Korea. Thirty mice were used for type 1 and type 2 diabetic model experiments respectively, out of which they were divided into Test and Control mice. Thirty mice were injected with streptozotocin (50 mg/kg) (Sigma, USA) for 5 days according to the Animal Models of Diabetic Complications Consortium (AMDCC). Another 30 mice were fed with a high fat diet with 60% kcal fat for 2 months before administrering the yogurt (Central Lab. Animal Inc. Japan). The test group mice were fed with the yogurt prepared using the *L. lactis* secreting peptide blocker and the control with the curd prepared with the wild type *L. lactis* strain. Yogurt was supplemented along with the water and food.

Yogurt preparation and administration

First, 10% milk was prepared by dissolving non fat dried milk powder in distilled water and pasteurized by heating at 66º for 50 minutes and then cooled immediately to 4ºC (Smith *et al.*, 1981). After cooling *L.lactis* MG1363, overnight grown culture was added aseptically, mixed and incubated at 37º for overnight and stored at 4ºC not more than 3 days.

Amylase inhibition assay

200 mg of mouse pancreas and duodenum samples along with the enzyme extraction buffer consisting protease inhibitor was homogenized and then centrifuged. The supernatant was used as the source of the amylase (Hokari *et al.,* 2003). *L. lactis* MG1363 was cultured overnight and culture supernatant was used as the source of PAMI. The amount of recombinant PAMI was calculated using the results obtained from immunoblotting. Amylase activity in pancreas and duodenum extract and its inhibition assay's were performed using Enzcheck Ultra Amylase Assay kit (Invitrogen Detection Technologies, USA). 2 mU/ml amylase enzyme from pancreatic and duodenum crude extract was kept constant and 0-250 µM PAMI was added and incubated for 30 minutes. As per the kit method fluorescent labeled starch substrate was added and the amylase activity was measured using fluorescent spectrophotometer VICTOR^3 (Perkin Elmer, USA).

Glucose measurements

Blood glucose level was measured at 10 day intervals. In Type1 mouse, the postprandial blood glucose level was measured immediately after feeding and in Type 2 mouse random blood glucose level was measured using Accu-Check Active (Roche, Germany). As per the Accu-Check Active kit method we used the 8 µl blood, puncturing the lateral tail vein using a sterilized needle.

Statistical methods

All results are presented as mean ±SE and were analyzed by student's t test and ANOVA using Microsoft excel. Values were considered statistically significant when p values were less than 0.05 or 0.01.

RESULTS

Cloning of PAMI in pVE5523 vector and transformation

To deliver the PAMI into the small intestine using *L.lactis* we used pVE5523 as the expression vector. *Sal1* and *EcoRV* restriction site of the plasmid and the synthetic gene was digested and then ligated and transformed into *E. Coli* DH5α competent cell. The plasmid preparation from *E.Coli* DH5α was again transformed into *L. lactis* competent cells by electroporator. As a result, the plasmid contains the entire structural gene of the PAMI which was confirmed by PCR and gene sequencing. The PCR results of the vector containing the PAMI are presented in Figure 1. Lanes 1, 2 and 4 represent the plasmid from *L. lactis* MG1363, *L. lactis* IL1403 and *E. Coli* DH5α. Figure 2 represents the fusion gene constructs with the PAMI gene.

Expression of PAMI in the genetically engineered *L. lactis*

PAMI expression in the recombinant *L. lactis* was examined using the dot-blotting assay. PAMI synthesized commercially was used as the positive control. To test specific expression of the PAMI, we obtained the antibodies synthesized commercially against PAMI in rabbits as described in 'materials and methods'. Dots 1, 2 and 3 in Figure 3 represent the control, *L. lactis* MG1363 and *L. lactis* IL1403 respectively. As shown in the figure no expression is observed in *L. lactis* IL1403 for some reason. However, expression was noticed in *L. lactis* MG1363. As per the chemi-doc image analysis we obtained approximately 1.2 µg/ml peptide synthesized, which was helpful to design the amylase inhibition study.

Amylase inhibition

After preparation of the crude extraction, amylase inhibition assay was performed. 2 mU/ml amylase was taken for this assay both in positive control and in bacterial suspension. Inhibition assay was performed both on pancreatic and duodenum amylase crude extracts. Figures 4.1 and 4.2 shows the inhibition of the amylase with recombinant PAMI and the positive control PAMI obtained commercially. There have been similar plots noticed both in the recombinant and the positive control.

Response of type 1 and type II diabetic mice models after oral administration of the yogurt prepared from *L. lactis* secreting recombinant PAMI

We used type 1 and type 2 diabetes mice models for the assay. The oral intake of yogurt by both

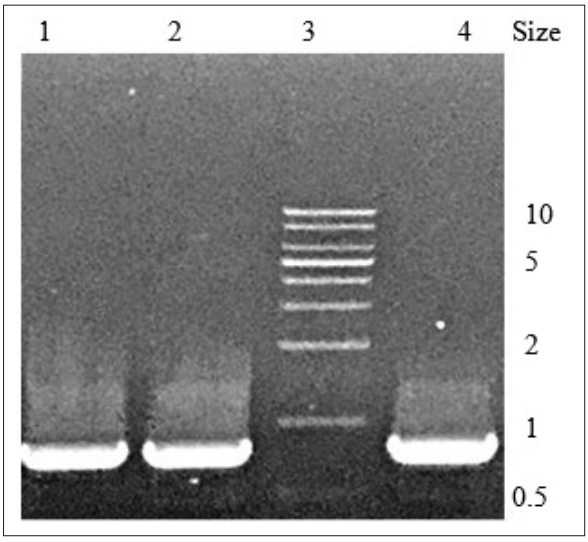

FIGURE 1 - *Electrophoregrams of PCR products from pVE5523 with PAMI gene. Lane 1, 2 and 4 corresponds to plasmid extracted from L. lactis MG1363, L. lactis IL1403 and E. Coli DH5α.lane 3 represents the marker.*

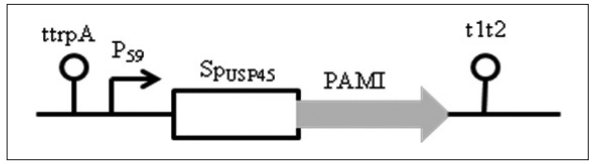

FIGURE 2 - *Fusion gene construct with PAMI gene. ttrpA, initiator; P_{59} lactococcal promoter; $SpUSP_{45}$ signal peptide; PAMI, proteinaceous amylase inhibitor gene; t1t2, terminator.*

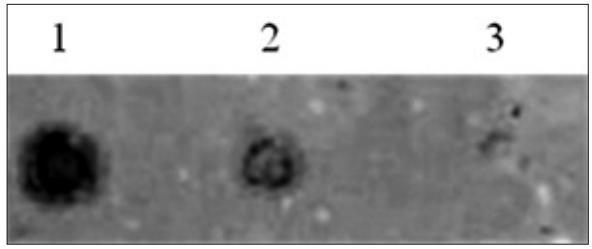

FIGURE 3 - *Expression of PAMI in L.lactis. 1) Control 2) L. lactis MG1363 and 3) L.lactis IL1403. There is no expression observed in L. lactis IL1403.*

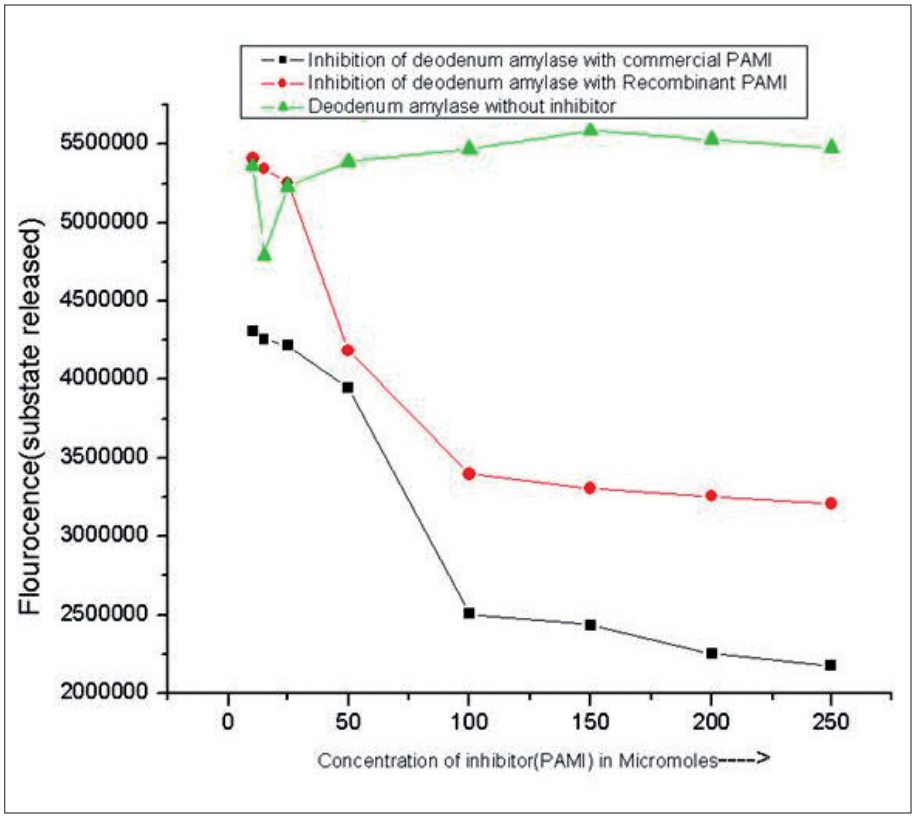

FIGURE 4.1 - *Amylase inhibition assay performed on crude extract of deodenum of mouse.*

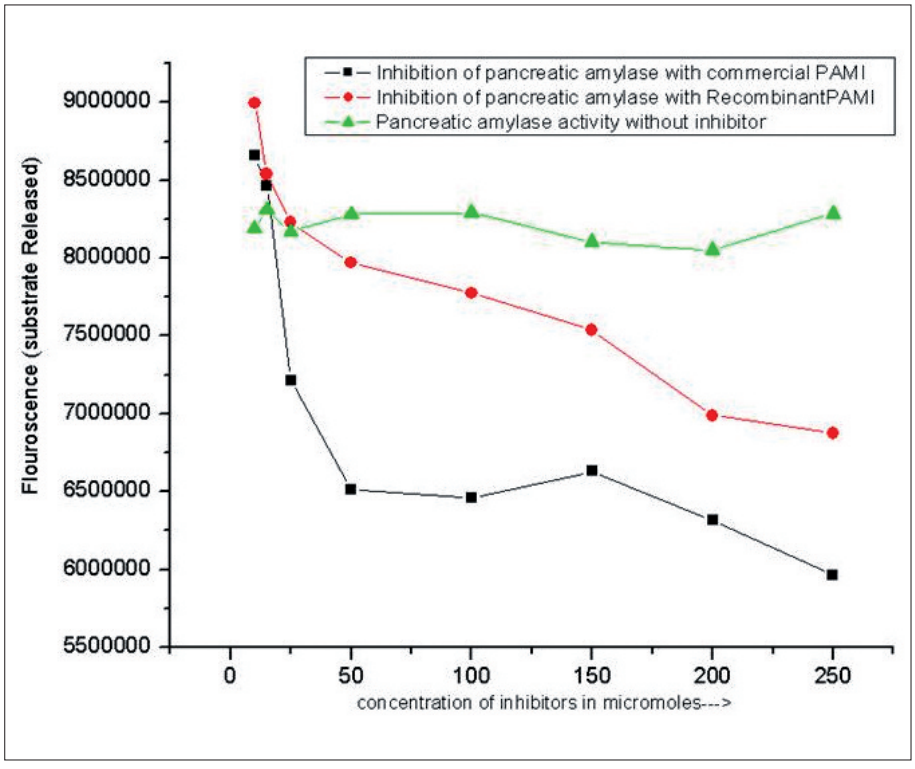

FIGURE 4.2 - *Amylase inhibition assay performed on crude extract of Pancreas of mouse.*

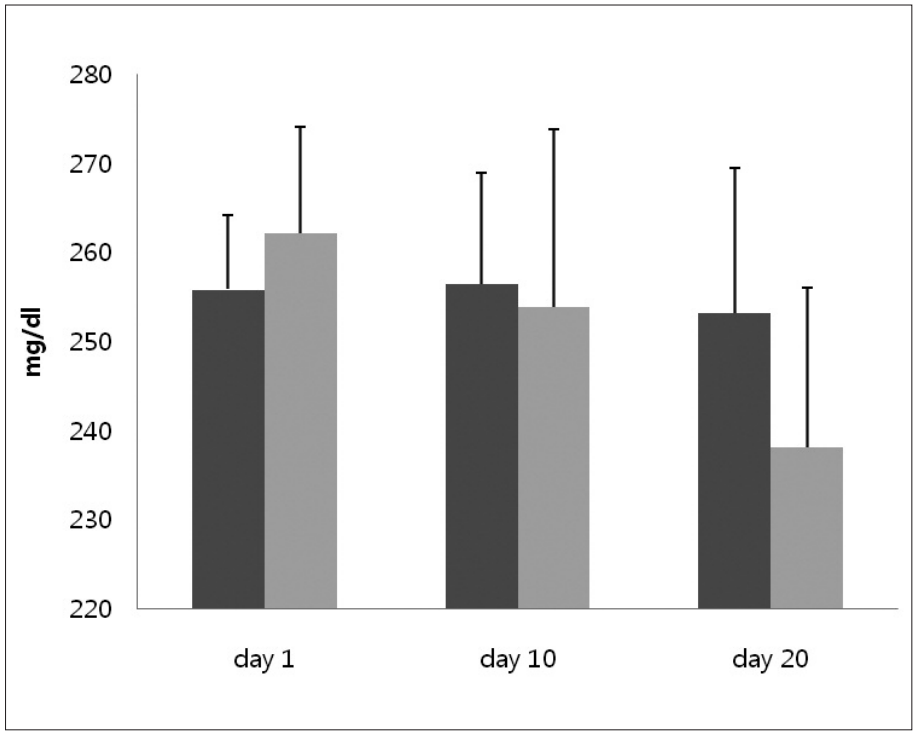

FIGURE 5.1 - *Fasting blood glucose level (mg/dl) in STZ induced Diabetic mouse: there has been decrease in the blood glucose level in the test group compared to the control group at day 20.*

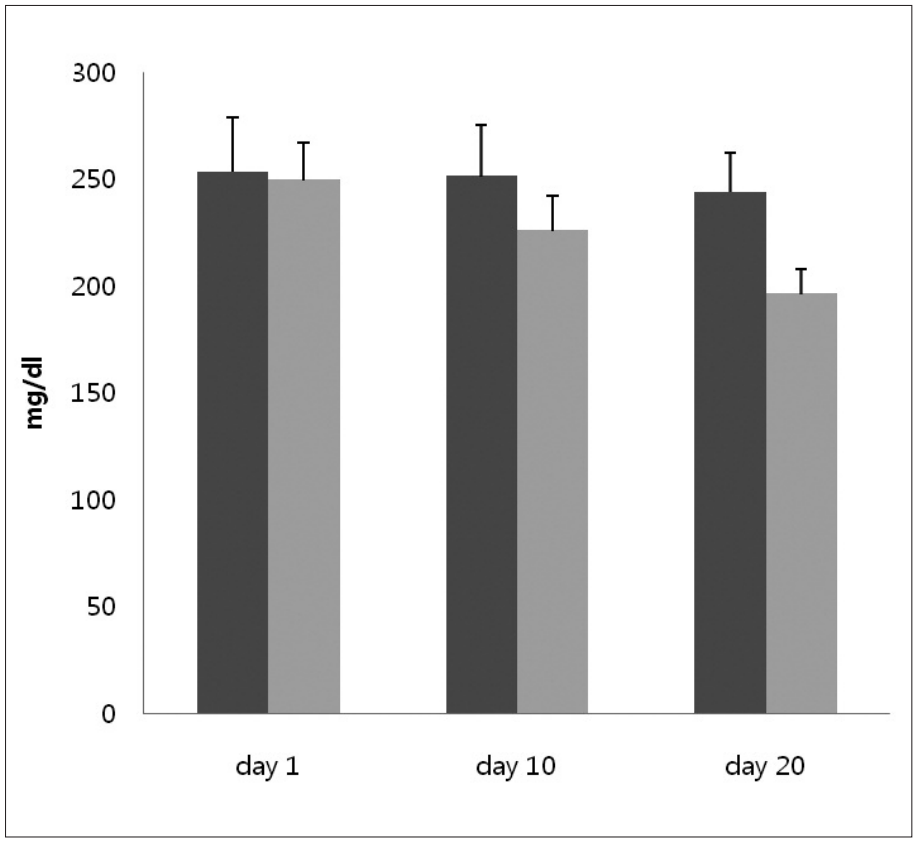

FIGURE 5.2 - *Postprandial Blood glucose level (mg/dl) in high fat fed diabetic mouse: Yogurt prepared with recombinant L. lactis expressing PAMI has been fed to both control and test. There is a gradual decrease in the blood glucose level in Day 20.*

test and control was 4.5 ml per day per each mouse. Fasting and postprandial blood glucose was measured at ten day intervals until 20 days. When the experiment started, in type 1 study the control and test both were around 255 mg/dl. As we continued feeding the yogurt there was a decrease in the blood glucose level on day 20 i.e. 239 mg/dl, whereas the control group remained the same (Figure 5.1).

Similarly, we conducted the experiment on type 2 mouse models. Type 2 mice were considered diabetic when the postprandial blood glucose level was around 250 mg%. As we continued feeding the yogurt at the end of the experiment there was a decrease in the blood glucose in test group i.e. 196.3 mg/dl (Figure 5.2). However, statistical data showed no significant decrease in the blood glucose level in test when compared to the control groups.

DISCUSSION

Numerous therapies have been used in the treatment of diabetes type 1 and type 2. Tight glycemic control using intensive insulin therapy was shown in the DCCT (Diabetes Control and Complication Trial) to reduce rates of microvascular complications in type 1 diabetes (The DCCT Research Group., 1993).

However, achieving and maintaining such control in type 1 diabetes using standard insulin therapy requires a high level of support and is associated with more hypoglycemia, increased weight gain and, in some patients, aggravation of cardiovascular risk factors including dyslipidaemia (Purnell *et al.,* 1998, Sibley *et al.,* 2003). In addition, stem cell therapy is said to be one of the most promising treatments in the near future (Carlos *et al.,* 2009).

However, the cost of such therapy can be expensive for the general population. Acorbose has been used for the treatment of type 2 diabetes. Acarbose not only inhibits disaccharidase activity, but also the activities of pancreatic and salivary -amylase. Unabsorbed polysaccharides in the intestine are broken down by enterobacteria and increase intestinal gas production. Therefore, the flatulence and abdominal bloating scores are likely to be higher after acarbose administration (Kazutaka *et al.,* 2010).

Our ultimate objective is to design a biodrug and check its response in streptozotocin-induced mouse and high fat fed diabetic model mouse. To use microorganisms as delivery vectors to the gastrointestinal tract, heterologous gene expression strategies have already been developed. The biodrug concept involves the use of orally administered recombinant microorganisms as a new drug delivery route to prevent or treat diseases. As a biodrug *L.lactis* offers several advantages with regard to genetic manipulation, culture facilities and low-cost production.

However, there was no significant decrease in the blood glucose level compared to the control group. The yogurt made by recombinant *L.lactis* will have no side-effects as this is a natural food (Hariom *et al.,* 2006). In this paper we described for the first time the construction of the plasmid having an insert of the 24 nucleotide, which was able to secrete the octa-peptide blocker. This strategy can be made more effective by constructing better expression vectors and promoters with an activity that is adjustable and adapted to the digestive environment. In addition, it mjust be ascertained if the long-term oral administration of this biodrug made by recombinant *L.lactis* can prove beneficial therapeutically.

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