Indian Journal of Biochemistry & Biophysics
Vol. 58, October 2021, pp. 408-415

Oral insulin delivery using artificial peptide

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Received 08 December 2019; *revised 03 September 2020*

The daily multiple insulin injection is the line of treatment for diabetes mellitus. As the oral insulin delivery mimics, the physiology of endogenous insulin secreted by pancreas. Recently, the search for suitable carrier to develop oral insulin delivery has been intensified. However, the carrier toxicity and very less bioavailability of insulin remains the major problem in the development of oral insulin delivery. Preparation of a non-covalent insulin-peptide complex using different peptide made of 16 to 20 L-amino acids studied to overcome the problem. The *in vitro* testing of insulin-peptide complex showed significant stability against the proteolytic enzyme. Whereas, in *in vivo* testing, the presence of 10-41% insulin in blood plasma observed after 30 to 60 min oral feeding of insulin-peptide complex. Results indicated that the peptide which showed moderate protection against pepsin and minor protection against trypsin and chymotrypsin has an important role in enhancing oral insulin bioavailability. However, the peptide which showed higher protection against trypsin and no protection against pepsin could not achieve significant oral insulin bioavailability.

Keywords: Bioavailability, Carrier toxicity, Insulin-peptide complex, Oral insulin delivery, Proteolytic enzyme resistance

Around four million people are dying per year due to diabetes mellitus and this disease may reach to 642 million people by 2040[1]. In general, diabetes mellitus is classified as Type 1 and Type 2. Type 1 diabetes mellitus is caused by an autoimmune destruction of insulin beta cells in the pancreas and is controlled by parenteral administration of insulin. Whereas, Type 2 diabetes is characterized by variable degrees of insulin resistance, impaired secretion of insulin and increased glucose production, and is generally controlled by oral antidiabetic agents. However, when these oral antidiabetic agents failed to reduce glycemia then these patients are also treated with insulin. Thus, insulin is the only effective therapeutic agent for the treatment of diabetes mellitus, which is being administered parenterally. Although from efficacy point of view, the subcutaneous administration of exogenous insulin is satisfactory route. However, this can result in peripheral hyperinsulinemia, which has been associated with hypertension, development of atherosclerosis, and increased insulin resistance in peripheral and muscle tissues. Additionally, the burden of daily injections can also result in a psychological discomfort, pain, high probability of infections, and local deposition of insulin leading to local lipodystrophy at the injection site[1]. However, the recent development of a small needle for insulin subcutaneous injection is almost painless, but the prospect of puncturing the skin still elicits significant psychological resistance in most of the patients. Another disadvantage of insulin subcutaneous injection is delayed absorption, which results in a relatively broad plasma insulin peak and a prolonged duration of action, potentially increasing the risk of hypoglycemia[2]. However, if oral route proven feasible for insulin delivery, it would greatly boost patient compliance for insulin. As when insulin given orally, it is directly channeled from the intestine to the liver and a high-level of insulin is reached in the portal blood, simulating the physiological secretion pattern of the pancreas[3].However, the carrier toxicity and very less bioavailability (therapeutic efficacy) of insulin are the major problems in oral delivery of insulin, which remains unsolved so far[4,5]. As insulin a 51 amino acid peptide cannot be orally administered because it is degraded by gastro intestinal tract (GIT) proteolytic enzymes[6]. Secondly, insulin is less absorbed by the intestinal epithelium mainly due to the large size and hydrophilicity of the molecule. Other routes such as the inhaled insulin, nasal, buccal, and transdermal administration of insulin have also been explored, but failed to achieve desired bioavailability because of very large

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absorption area. Therefore, there is a need of new approach to overcome the problem of carrier toxicity and bioavailability.

Recently, many novel techniques have been developed to optimize the preparation of oral insulin delivery carriers. Nano-particles as a carrier for oral insulin delivery has been widely studied[5,7-10]. Similarly, other types of carrier for oral insulin delivery also extensively studied such as natural polymers[11,12], liposomes[13], and polypeptide-based polyelectrolyte[14]. The new approach such as modification of carrier surface charge is also reported to enhance insulin bioavailability[1,15]. However, the problem of toxicity associated with the carrier and insulin bioavailability remains by and large unsolved so far[4]. Thus, the present study proposed the development of peptide made of L-amino acids to overcome the carrier toxicity problem. As the degradation product of the peptide will be L-amino acids and it can be used for the protein synthesis. Similarly, the development of a non-covalent insulin-peptide complex that avoids any chemical modification of insulin. Thus, this may result in the protection of insulin natural bioactive form and achieve desired bioavailability.

Materials and Methods

P*reparation of* insulin-peptide complex

The different peptides comprising of 16-20 L-amino acids having positive and/or negative charge with hydrophobic and aromatic or cyclic character were design and prepared *as described elsewhere*[16]. The purity of all the peptide was around 95% and the N- terminal and C-terminal was protected with amide and acetyl group respectively. All the chemicals used were of ACS grade otherwise specified. The non-covalent insulin-peptide complex was prepared by mixing insulin and peptide. Insulin was purchased as recombinant human insulin from Sigma-Aldrich Chemical Company (St. Louis, MO). In a glass tube insulin (15.0 mg) was dissolved in 3.0 mL solution of 0.01 N HCl pH 2.0. Similarly, 15.0 mg of peptide was dissolved in 3.0 mL solution of 0.01 N HCl pH 2.0. Thereafter, 1.0 mL solution of insulin was distributed in three glass test tubes and to each tube 1.0 mL solution of peptide was added and the mixture was allowed to stabilize. Then, the 2.0 mL mixture of insulin and peptide present in each tube was subjected to dry using Thermo Scientific Speed Vac. The dried mixture was termed as insulin-peptide complex and stored at −20°C till further use.

Verification of complex formation

The morphological verification of insulin-peptide complex was carried out using s*canning electron* microscopy (SEM). The verification of chemical groups present in the insulin-peptide complex was studied by performing fourier-transform infrared spectroscopy (FTIR). The insulin, peptide and insulin-peptide complex were scanned using Quanta 200 3D s*canning electron* microscope and standard procedures were used for preparation of samples. *FTIR of* insulin, peptide and insulin-peptide complex was obtained using Perkin Elmer FTIR spectrometer in the wavenumber range 500-4000 cm^{-1}. Standard procedures were used for preparation of samples and the respective spectrum is given as it is obtained from the instrument.

In vitro testing

The *in vitro* testing of insulin-peptide complex was carried out to verify the protection of insulin after the hydrolysis of insulin-peptide complex by the proteolytic enzyme. such as pepsin, trypsin, and chymotrypsin (Sigma-Aldrich). The following procedure was used to carry out *in vitro* testing:

Complex preparation -insulin (18.0 mg) was dissolved in 18.0 mL solution of 0.01 N HCl pH 2.0. Similarly, 18.0 mg of peptide was dissolved in 18.0 mL solution of 0.01 N HCl pH 2.0. Thereafter, 1.0 mL solution of insulin was distributed in eighteen glass test tubes and to each tube 1.0 mL solution of peptide was added and the mixture was allowed to stabilize. Then, the 2.0 mL mixture of insulin and peptide present in each tube was subjected to dry using Thermo Scientific Speed Vac. The dried mixture was termed as insulin-peptide complex and stored at −20°C till further use.

Enzyme preparation - pepsin (10.0 mg) was dissolved in 20.0 mL solution of 0.01 N HCl pH 2.0 and trypsin, chymotrypsin 10.0 mg each was dissolved in 20.0 mL solution of PBS pH 7.4. Each enzyme solution was divided in to two test tubes as 10.0 mL in each test tube. One test tube carrying 10.0 mL enzyme solution was termed as active enzyme. Another testtube carrying 10.0 mL enzyme solution was kept in a boiling water bath for 5 minutes to inactivate the enzyme and termed as inactivated enzyme.

Reaction mixture for enzyme hydrolysis:1. Reaction mixture for pepsin -Pepsin test (The dried insulin-peptide complex + 1.5 mL 0.01 N HCl pH 2.0 + 0.5 mL active pepsin); Pepsin control (The dried

insulin-peptide complex + 1.5 mL0.01 N HCl pH 2.0 + 0.5 mL inactivated pepsin). 2. Reaction mixture for trypsin -Trypsin test (The dried insulin-peptide complex + 1.5 mL solution of PBS pH 7.4. + 0.5 mL active trypsin); Trypsin control (The dried insulin-peptide complex + 1.5 mL solution of PBS pH 7.4. + 0.5 mL inactivated trypsin); 3. Reaction mixture for chymotrypsin -Chymotrypsin test (The dried insulin-peptide complex + 1.5 mL solution of PBS pH 7.4. + 0.5 mL active chymotrypsin); Chymotrypsin control (The dried insulin-peptide complex + 1.5 mL solution of PBS pH 7.4. + 0.5 mL inactivated chymotrypsin). All the reaction mixture for each enzyme was prepared in triplicate. The hydrolysis was carried out by incubating reaction mixture in a water bath at 37°C for one hour. After one hour the sample was removed, and the verification of insulin protection after the hydrolysis of insulin-peptide complex by the proteolytic enzyme was carried out using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was carried out as per the procedure described by Laemmli[17], using a 15.0% stacking gel and 4.0% resolving gel and gels were stained by Coomassie brilliant blue G-250 (Sigma-Aldrich). The concentration of insulin in the sample loaded on the SDS-PAGE was 12.0 µg before hydrolysis. Thus, 12.0 µg insulin was loaded on the SDS-PAGE as a control for comparison.

Complex preparation for *in vivo* testing

Complex preparation for single peptide: The non-covalent insulin-peptide complex for single peptide such as peptide A, peptide B, peptide C, and peptide D was prepared by mixing insulin and peptide. In a glass test tube insulin (25.0 mg) was dissolved in 5.0 mL solution of 0.01 N HCl pH 2.0. Similarly, 25.0 mg of respective peptide was dissolved in 5.0 mL solution of 0.01 N HCl pH 2.0. Thereafter, 1.0 mL solution of insulin was distributed in five glass test tube and then 1.0 mL solution of peptide was added to each glass test tube and the mixture was allowed to stabilize. Then, the 2.0 mL mixture of insulin and peptide present in each tube was subjected to dry using Thermo Scientific Speed Vac. The dried mixture was termed as insulin-peptide complex and stored at −20°C till further use.

Complex preparation for double peptide: The non-covalent insulin-peptide complex fordouble peptide such as peptide A+ peptide B (PAPB) and peptide C+ peptide D (PCPD) was prepared by mixing insulin and peptide. In a glass tube insulin (25.0 mg) was dissolved in 5.0 mL solution of 0.01 N HCl pH 2.0. Similarly, 12.5 mg of peptide A /peptide C and 12.5 mg of peptide B / peptide D was dissolved in 5.0 mL solution of 0.01 N HCl pH 2.0. Thereafter, 1.0 mL solution of insulin was distributed in five glass test tube and then 1.0 mL solution of peptide was added to each glass test tube and the mixture was allowed to stabilize. Then, the 2.0 mL mixture of insulin and peptide present in each tube was subjected to dry using Thermo Scientific Speed Vac. The dried mixture was termed as insulin-peptide complex and stored at −20°C till further use.

In vivo testing

The *in vivo* testing was carried out at National Laboratory Animal Center, CSIR-CDRI, Lucknow, India to verify the presence of bioactive insulin in blood plasma after feeding the insulin-peptide complex through gastro intestinal tract. The dried mixture of insulin-peptide complexwas reconstituted in pH 2.5 solution and administered orally using curved metal gavage needles. Male Sprague Dawley rats (200±20 g) available at the National Laboratory Animal Center, CSIR-CDRI, Lucknow, India were used for the study. The work was conducted in accordance with the approval of Institutional Animal Ethics Committee (IAEC). The animals were housed in polypropylene cages in the animal house, with temperature 23±2°C, relative humidity 60-70%, 12 h day-night cycle, provided standard pellet diet and drinking water. At the day of experiment animals were fasted (*ad libitum*) for 6 h. After fasting, blood was collected to get the basal (0 min) insulin level from all the animals (n=27). The reconstituted Insulin-Peptide complex was administered orally (5 mg/kg) to animals (n=3 each) and blood from tail tip at 30 and 60 min of post-treatment was collected. Control group was treated with recombinant human insulin (5 mg/kg; n=3) purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). The blood was centrifuged at 5000 rpm for 5 min at cold and the plasma was collected for the measurement of insulin using ELISA kit. Level at basal was considered 100% and calculation was made to check the availability after treatment.

Results and Discussion

Peptide design

Many efforts have been going on to develop a suitable carrier for oral insulin delivery[1,5,7-15]. However, carrier toxicity and low bioavailability

remains unsolved problem in oral insulin delivery so far[4]. Particularly, the problem of carrier toxicity becomes more evident in case of diabetes mellitus, as it requires lifelong multi-dose treatment to control it. In such a case, the prediction of long-term effect of carrier toxicity becomes difficult. Thus, the carrier has to be as close as to the natural compound that human body is used to. This has prompted to use peptide made of L-amino acids as a carrier for oral insulin delivery. In our previous work, it was found that the peptide can significantly protect degradation of antisense oligdeoxynucleotides from the nuclease[18]. However, not much work is reported regarding the use of peptide in the protection of insulin from proteolytic enzymes. As the protection of insulin from degradation by the proteolytic enzymes is the basic criteria in the development of carrier suitable for oral insulin delivery. However, some peptide related work has been reported in the development of oral insulin delivery. The use of polypeptide-based polyelectrolyte complexeshas been reported for oral insulin delivery, but the raw material showed some toxicity[14].In another report, ~17% relative bioavailability was observed when glycyl-glycine and alanyl-alanine used to prepare conjugate nanoparticles of trimethyl chitosan for oral insulin delivery[9]. Thus, in the present study, the strategy to protect insulin from proteolytic enzyme and pH shift of stomach and intestine was adapted in designing peptide. As the insulin molecule has amino acids carrying positive, negative charge and hydrophobic character. Secondly, the isoelectric point of insulin is around pH 5.3, this means the overall charge on insulin molecule may be positive in stomach and negative in the intestine. Thus, the peptide having negative and/or positive charge and hydrophobic character may form the non-covalent bonding with insulin molecule. This may protect insulin from pH shift and proteolytic enzyme by shielding the site of action available on the insulin molecule. Considering all these factors, the different peptide was designed and used to verify their role in the protection of insulin from proteolytic enzyme.

***In vitro* testing**

The *in vitro* testing was carried out to verify the protection of insulin after the hydrolysis of insulin-peptide complex by the proteolytic enzyme such as pepsin, trypsin, and chymotrypsin. The proteolytic enzyme such as pepsin, trypsin, and chymotrypsin were selected for the testing because these enzymes are the principal proteases in the human digestive system. Figure 1 shows the band obtained for different proteolytic enzyme (test) and insulin as a control. The less or more degree of protection

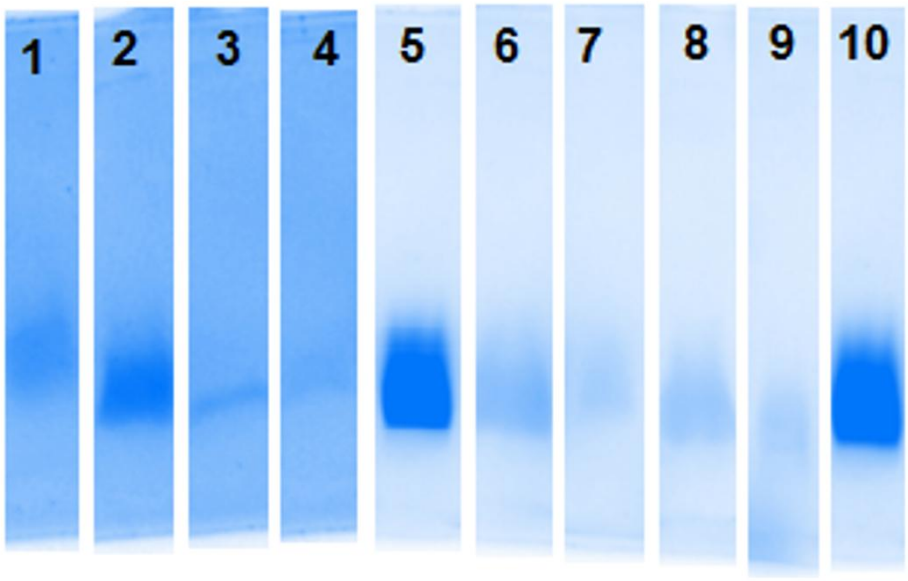

Fig. 1 — SDS-PAGE of insulin-peptide complex treated with proteolytic enzyme. [1- Insulin-peptide A complex treated with Pepsin; 2 - Insulin-peptide B complex treated with Pepsin; 3 - Insulin-peptide B complex treated with Trypsin; 4 - Insulin-peptide B complex treated with Chymotrypsin; 5 - Insulin-peptide C complex treated with Trypsin; 6: Insulin-peptide C complex treated with Chymotrypsin; 7 - Insulin-peptide D complex treated with Pepsin; 8 - Insulin-peptide D complex treated with Trypsin; 9 - Insulin-peptide D complex treated with Chymotrypsin; 10 – Insulin (12.5 µg)]

obtained against respective proteolytic enzyme can be seen and understood by comparing the intensity of the respective band (1-9) with band number 10 obtained for insulin. Insulin-peptide complex made of four peptides such as peptide A (Theoretical pI/Mw: 10.70 /2297.83 Da); peptide B (Theoretical pI/Mw: 10.70/ 2137.73Da); peptide C (Theoretical pI/Mw: 3.46/ 2143.38 Da) and peptide D (Theoretical pI/Mw: 4.71/ 2303.48Da) showed protection against one or more proteolytic enzyme. Comparatively, ~10% with peptide A against pepsin; ~40%, ~5%, ~2% with peptide B against pepsin, trypsin and chymotrypsin respectively; ~100% and ~10% with peptide C against trypsin and chymotrypsin, respectively; ~2%, ~5%, ~3% with peptide D against pepsin, trypsin and chymotrypsin respectively. Peptide A showed some resistance against pepsin, but no resistance against trypsin and chymotrypsin. All the control band lookalike as band number 5, which was observed for insulin-peptide C complex treated with trypsin.

At pH 2.0 peptide A and insulin has an overall positive charged. Thus, interaction between hydrophobic amino acid of peptide A and insulin might have given some degree of protection from pepsin, but not against trypsin and chymotrypsin. As pepsin cleavespeptide bonds between aromatic amino acids whereas, chymotrypsin cleaves aromatic amino acids. Trypsin cleaves peptide chains mainly at the carboxyl side of the amino acidslysine or arginine, except when either is followed by proline. However, peptide B showed comparatively moderate protection against pepsin and minor protection against trypsin and chymotrypsin. Peptide B has comparatively less positive charge than peptide A and it contains proline that can cause a kink. Higher protection against trypsin and moderate protection against chymotrypsin was obtained with peptide C. As at pH 7.4 peptide C and insulin has overall negative charge. This might have protected carboxyl side of lysine or arginine effectively, but moderately protected aromatic amino acids. However, peptide D has showed marginal protection againstpepsin, trypsin and chymotrypsin. Peptide D has overall negative charge and it contains proline that might have given some degree of protection. However, it was reported that the negatively charged insulin was most strongly attracted to a negatively charged interface than positively charged[19]. Thus, it appears that the net charge of insulin may play a secondary role in the interaction with a charged surface. Thus, it is difficult to give clear explanation as to why some peptide protected insulin and some failed. However, the strategy adapted in designing the peptide appears to be a successful. As the results clearly showed the protection of insulin-peptide complex from total degradation by proteolytic enzymeat 37°C for one h. Thus, the peptide A, peptide B, peptide C and peptide D selected to carry out *in vivo* testing.

Verification of complex formation

The verification of insulin-peptide complex was carried out using SEM and FTIR. The pattern obtained for all the insulin-peptide complex was almost similar. Figure 2 shows themorphology of insulin, peptide and insulin-peptide complex obtained on SEM. Figure 2 clearly shows the morphology of insulin, peptide and insulin-peptide complex is totally different. However, the morphology of insulin-peptide complex appears well-organized as compare to insulin and peptide. This suggests the reorganization of insulin and peptide molecules after the complex formation. This indicates the formation of non-covalent linkages between insulin and peptide that results in the formation of well-organized structure. Figure 3 shows the chemical groups present in the insulin, peptide and insulin-peptide complex analyzed by the FTIR. The chemical groups present in the form of peaks related to insulin and peptide are almost present in the insulin-peptide complex. These all the observation strongly suggest the formation of a stable non-covalent insulin-peptide complex.

***In vivo* testing**

The four peptides, which showed some positive results against proteolytic enzyme (3.2) were selected to carry out *in vivo* testing. Figure 4 shows the availability of bioactive insulin in blood plasma after feeding insulin-peptide complex through GIT. In comparison with control (0 min: 100 ± 8.53%),peptide B (30 min: 117.37 ± 13.10%; 60 min: 130.61 ± 0.48%), peptide C (30 min: 110.48 ± 8.71%), peptide PAPB (30 min: 141.22 ± 21.41%) and peptide PCPD (30 min: 116.70 ± 1.48%) showed increase in plasma insulin level after the GIT administration. After 60 min of GIT administration of insulin-peptide B complex, almost 30% stable increase in plasma insulin level was obtained. This appears to be a significant improvement in an oral insulin bioavailability. On the contrary, 5.2% relative pharmacological availability of the insulin was observed after oral insulin delivery *via* surface hydrophilic modification of chitosan copolymer based

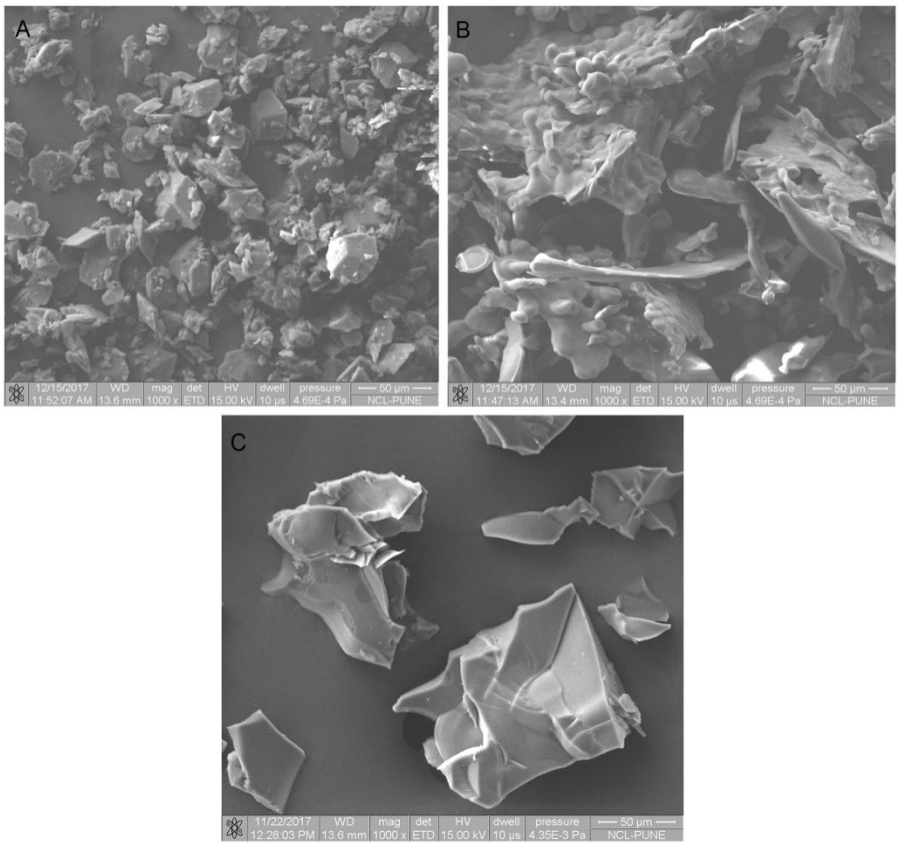

Fig. 2 — Scanning electronmicroscopy image of (A) Insulin; (B) Peptide; and (C) Insulin-peptide complex

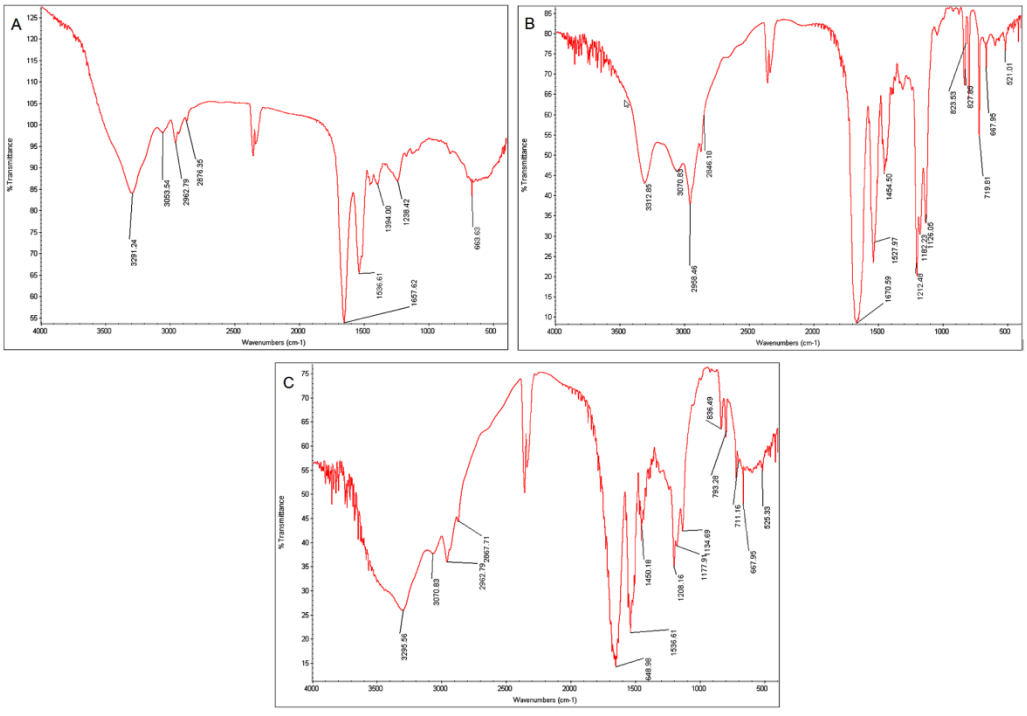

Fig. 3 — Fourier-transform infrared spectroscopy spectra of (A) insulin; (B) Peptide and (C) Insulin-peptide complex. Figures are given as it is obtained from the instrument without any modification

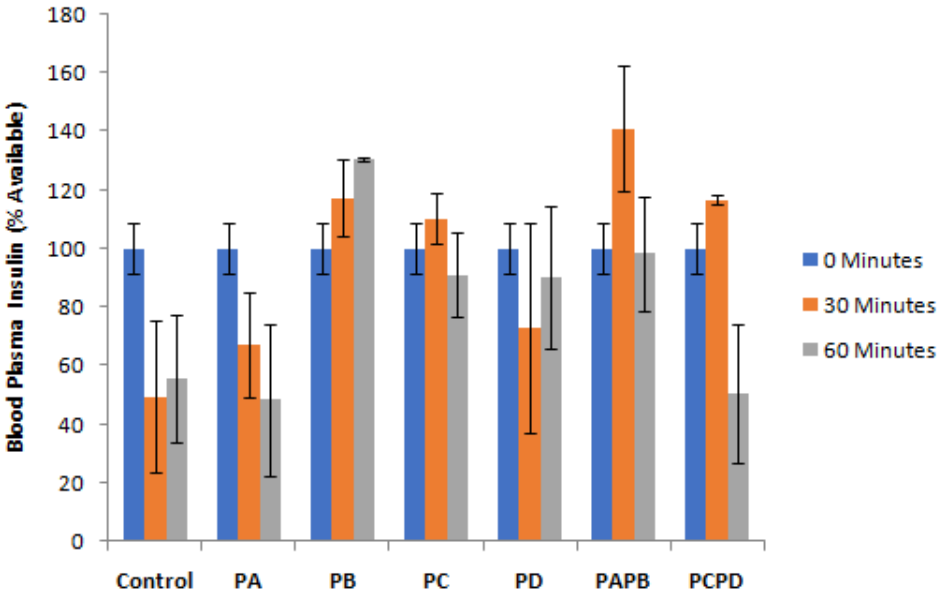

Fig. 4 — Availability of insulin in blood plasma after feeding insulin-peptide complex through gastro intestinal tract. [PA- Insulin-peptide A complex; PB - Insulin-peptide B complex; PC - Insulin-peptide C complex; PD- Insulin-peptide D complex; PAPB - Insulin-peptide A complex + Insulin-peptide B complex; PCPD - Insulin-peptide C complex + Insulin-peptide D complex; Control – Insulin]

self-assembly polyelectrolyte nanocomplex[1].The nanoparticles made of poly (d, lactic-co-glycolic) acid showed less than 20% insulin bioavailability[15]. Whereas, 15.9% insulin oral bioavailability was reported with deoxycholic acid-modified nanoparticles[20]. The oral administration of glycyl-glycine and alanyl-alanine conjugate nanoparticles of trimethyl chitosan demonstrated 17.19% and 15.46% relative insulin bioavailability increase respectively[9]. In summary, the strategy adapted showed almost 40% oral insulin bioavailability. On the contrary, approximately 50% bioavailability was reported when insulin solutions injected into the dorsal subcutaneous tissues of rats[21].

Conclusion

The results clearly showed that the strategy used in the present study has a high potential to achieve oral insulin bioavailability comparable with subcutaneous injection[21], or perhaps better. As the 130.61 ± 0.48% and 141.22 ± 21.41% oral insulin bioavailability was obtained with peptide B and peptide A+B respectively. This appears that the peptide which showed moderate protection against pepsin and minor protection against trypsin and chymotrypsin has an important role in enhancing oral insulin bioavailability. Comparatively, the peptide which showed higher protection against trypsin and no protection against pepsin could not achieve significant oral insulin bioavailability. The further study such as optimization of peptide concentration and peptide combination in the formation of insulin-peptide complex will certainly evolve a safe, effective and marketable oral insulin for the treatment of diabetes mellitus.

Acknowledgement

The author gratefully acknowledges Science and Engineering Research Board (A Statutory body of DST, Government of India), New Delhi, for financial support (SB/EMEQ-147/2013). The author also thanks Dr. Jiaur R. Gayen, and his team (CSIR-Central Drug Research Institute, Lucknow, India) for carrying out in-vivo study and project assistants for their technical support.

Conflict of interest

All authors declare no conflict of interest.

References

1 Liu C, Kou Y, Zhang X, Dong W, Cheng H & Mao S, Enhanced oral insulin delivery *via* surface hydrophilic modification of chitosan copolymer based self-assembly polyelectrolyte nanocomplex. *Int J Pharm*, 554 (2019) 36.

2 Vajo Z, Fawcett J & Duckworth WC, Recombinant DNA technology in the treatment of diabetes: Insulin Analogs. *Endocr Rev*, 22 (2001) 706.

3 Gedawy A, Martinez J, Al-Salami H & Dass CR, Oral insulin delivery: existing barriers and current counter-strategies. *J Pharm Pharmacol*, 70 (2018) 197.

4 Han Y, Gao Z, ChenL, KangL, Huang W, Jin M, Wang Q & Bae YH, Multifunctional oral delivery systems for

enhanced bioavailability of therapeutic peptides/proteins. *Acta Pharm Sin B*, 9 (2019) 902.

5 Zhang Y, Zhang L, Ban Q, Li J, Li CH & Guan YQ, Preparation and characterization of hydroxyapatite nanoparticles carrying insulin and gallic acid for insulin oral delivery. *Nanomedicine*, 14 (2018) 353.

6 Alai MS, Lin WJ & Pingale SS, Application of polymeric nanoparticles and micelles in insulin oral delivery. *J Food Drug Anal*, 23 (2015) 351.

7 Wong CY, Al-Salami H & Dass CR, Potential of insulin nanoparticle formulations for oral delivery and diabetes treatment. *J Control Release*, 264 (2017) 247.

8 Hu Q & Luo Y, Recent advances of polysaccharide-based nanoparticles for oral insulin delivery. *Int J Biol Macromol*, 120 (2018) 775.

9 Jafary ON, Bahari JN, Dehpour AR, Partoazar A, Rafiee TM & Dorkoosh F, *In vitro* and *in vivo* cytotoxicity and efficacy evaluation of novel glycyl-glycine and alanyl-alanine conjugates of chitosan and trimethyl chitosan nano-particles as carriers for oral insulin delivery. *Int J Pharm*, 535 (2018) 293.

10 Sudhakar S, Chandran S V, Selvamurugan N & Nazeer RA, Biodistribution and pharmacokinetics of thiolated chitosan nanoparticles for oral delivery of insulin *in vivo*. *Int J Biol Macromol*, 150 (2020) 281.

11 Nur M & Vasiljevic T, 2017. Can natural polymers assist in delivering insulin orally?. *Int J Biol Macromol*, 103 (2017) 889.

12 Vasconcelos Silva EL, Oliveira ACJ, Patriota YBG, Ribeiro AJ, Veiga F, Hallwass F, Silva-Filho EC, da Silva DA, Soares MFR, Wanderley AG & Soares-Sobrinho JL, Solvent-free synthesis of acetylated cashew gum for oral delivery system of insulin. *Carbohydr Polym*, 207 (2019) 601.

13 Wong CY, Al-Salami H & Dass CR, Recent advancements in oral administration of insulin-loaded liposomal drug delivery systems for diabetes mellitus. *Int J Pharm*, 549 (2018) 201.

14 Jeong YJ, Lee DY, Choe K, Ahn H, Kim P, Park JH & Kim YC, Polypeptide-based polyelectrolyte complexes overcoming the biological barriers of oral insulin delivery. *J Ind Eng Chem*, 48 (2017) 79.

15 Czuba E,Diop M, Mura C, Schaschkow A, Langlois A, Bietiger W, Neidl R, Virciglio A, Auberval N, Julien-David D, Maillard E, Frere Y, Marchioni E, Pinget M & Sigrist S, Oral insulin delivery, the challenge to increase insulin bioavailability: Influence of surface charge in nanoparticle system. *Int J Pharm*, 542 (2018) 47.

16 Adikane HV, World Patent, WO/2020/026272 (to Council of Scientific and Industrial Research, New Delhi, India) 06 February 2020.

17 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227 (1970) 680.

18 Wang M, Adikane HV, Duhamel J & Chen P, Protection of oligodeoxynucleotides against nuclease degradation through association with self-assembling peptides. *Biomaterials*, 29 (2008) 1099.

19 Jeworrek C, Hollmann O, Steitz R, Winter R & Czeslik C, Interaction of IAPP and insulin with model interfaces studied using neutron reflectometry. *Biophys J*, 96 (2009) 1115.

20 Fan W, Xia D, Zhu Q, Li X, He S, Zhu C, Guo S, Hovgaard L, Yang M & Gan Y, Functional nanoparticles exploit the bile acid pathway to overcome multiple barriers of the intestinal epithelium for oral insulin delivery. *Biomaterials*, 151 (2018) 13.

21 Tokihiro K, Arima H, Tajiri S, Irie T, Hirayama F & Uekama K, Improvement of subcutaneous bioavailability of insulin by sulphobutyl ether β-cyclodextrin in rats. *J Pharm Pharmacol*, 52 (2000) 911.