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DPP IV resistance and insulin releasing activity of a novel di-substituted analogue of glucose-dependent insulinotropic polypeptide, (Ser^2–Asp^{13})GIP

V.A. Gault[a*], N. Irwin[a], P. Harriott[b], P.R. Flatt[a], F.P.M. O'Harte[a]

[a] *School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, Northern Ireland, UK*
[b] *Centre for Peptide and Protein Engineering, School of Biology and Biochemistry, The Queen's University of Belfast, Medical Biology Centre, Belfast BT9 7BL, Northern Ireland, UK*

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Abstract

Structure–function studies suggest that preservation of the N-terminus and secondary structure of glucose-dependent insulinotropic polypeptide (GIP) is important for biological activity. Therefore, a novel di-substituted analogue of GIP, (Ser^2–Asp^{13})GIP, containing a negatively charged Asp residue in place of an Ala in position 13, was synthesised and evaluated for in vitro biological activity. Incubation with dipeptidyl peptidase IV (DPP IV) showed the half-lives of GIP and (Ser^2–Asp^{13})GIP to be 2.3 and >4 h, respectively. Insulin releasing studies in clonal pancreatic BRIN-BD11 cells demonstrated that (Ser^2–Asp^{13})GIP (10^{-12} to 10^{-7} mol/l) was significantly less potent (60–90%; $P<0.05$ to $P<0.001$) than native GIP. The peptide failed to display antagonistic properties as it did not significantly alter insulin secretion when incubated in the presence of GIP (10^{-7} mol/l). These results demonstrate that despite increased resistance to DPP IV, substituting Ala in position 13 with a negatively charged Asp, thus producing the di-substituted analogue (Ser^2–Asp^{13})GIP, significantly reduces biological activity, most likely due to modifications within the secondary structure.
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1. Introduction

Glucose-dependent insulinotropic polypeptide (GIP) is a potent insulin releasing hormone of the entero-insular axis (Creutzfeldt, 2001). The explicit pre-requisite of a hyperglycaemic environment in order for incretin-induced insulin release to occur, has led to recent interest in GIP as a novel therapy for type 2 diabetes (Hinke et al., 2002; O'Harte et al., 2000).

The widely distributed serine protease, dipeptidyl-peptidase IV (DPP IV), has been shown to be a key physiological regulator of incretin activity (Deacon et al., 2001; Kieffer et al., 1995). This intrinsic degradation of GIP results in the formation of the truncated metabolite GIP(3–42), which is now believed to operate as a GIP receptor antagonist (Flatt et al., 2001). Consequently, DPP IV-mediated degradation and subsequent production of GIP(3–42), greatly reduces the biological activity of GIP (Kieffer et al., 1995), therefore indicating the importance of an intact N-terminus in maintaining biological activity.

More recent studies examining the bioactive domain(s) of the GIP molecule have indicated the significance of the amino acids located within residues 1–14 (Hinke et al., 2001). Furthermore, computer-assisted secondary structure analysis of GIP has predicted that an alpha-helical structure exists between residues 10 and 29 (Hinke et al., 2001). Therefore, it was hypothesised that preservation of this helical structure is important for biological activity. Previous studies have determined that substitution of Ala^2 with Ser ((Ser^2)GIP) leads to increased DPP IV resistance and enhanced biological activity in vitro (Gault et al., 2001). Therefore, we have synthesised a novel di-substituted GIP analogue,

* Corresponding author. Tel.: +44-28-70324313; fax: +44-28-70324965
E-mail address: va.gault@ulster.ac.uk (V.A. Gault).

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substituted at both Ala^2 and Ala^{13} with Ser and Asp, respectively. Novel substitution of a neutral Ala^{13} with the negatively charged Asp residue will ascertain the importance of conserving the secondary structure in regulating biological activity.

2. Materials and methods

2.1. Chemicals

High-performance liquid chromatography (HPLC) grade acetonitrile was obtained from Rathburn (Walkersburn, Scotland). Sequencing grade trifluoroacetic acid (TFA) and DPP IV were obtained from Aldrich (Poole, Dorset, UK). Fmoc-protected amino acids were from Calbiochem Novabiochem (Beeston, Nottingham, UK). RPMI 1640 tissue culture medium, foetal bovine serum, penicillin and streptomycin were all purchased from Gibco (Paisley, Strathclyde, UK). Water used in these experiments was purified using a Milli-Q Water Purification System (Millipore, Milford, MA, USA). All other chemicals used were of the highest purity available.

2.2. Peptide synthesis

GIP and (Ser^2–Asp^{13})GIP were sequentially synthesised on an Applied Biosystems automated peptide synthesiser (model 432 A) using standard solid-phase Fmoc procedure, starting from an Fmoc-Gln-Wang resin. Following cleavage from the resin by TFA: water, thioanisole, ethanedithiol (90:2.5:5:2.5, a total volume of 20 ml/g resin), the resin was removed by filtration and the filtrate volume decreased under reduced pressure. Dry diethyl ether was slowly added until a precipitate was observed. The precipitate was collected by low-speed centrifugation, resuspended in diethyl ether and centrifuged again, the procedure being repeated five times. The resulting pellets were then dried in vacuo and judged pure by reversed-phase HPLC on a Waters Millenium 2010 chromatography system (Software version 2.1.5) and the peptides subsequently characterised using matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry.

2.3. MALDI-TOF mass spectrometry

Samples for MALDI-TOF analysis (1 μl) were mixed with 0.5 μl of a matrix solution (10 mg/ml solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/ethanol (1/1)), placed on one well of a 100-well stainless steel sample plate and allowed to dry at room temperature. The mass spectra were recorded using a Voyager-DE BioSpectrometry Workstation (PerSeptive Biosystems, Framingham, MA, USA). Masses were recorded as a mass-to-charge (m/z) ratio against relative peak intensity, and compared with theoretical values.

2.4. Degradation of GIP and (Ser^2–Asp^{13})GIP by DPP IV

GIP and (Ser^2–Asp^{13})GIP were incubated in vitro at 37 °C for 0, 2 and 4 h in 50 mmol/l triethanolamine–HCl (pH 7.8; final peptide concentration 2 mmol/l) with purified DPP IV (5 mU), which is representative of the normal physiological degradation process with GIP. The enzymatic reactions were stopped by the addition of 10 μl of 10% (v/v) TFA/water. The terminated reaction products were then applied to a Vydac C-18 column (4.6 × 250 mm) and the major degradation fragment GIP(3–42) separated from intact GIP or (Ser^2–Asp^{13})GIP. The column was equilibrated with 0.12% (v/v) TFA/water at a flow rate of 1.0 ml/min. Using 0.1% (v/v) TFA in 70% acetonitrile/water, the concentration of acetonitrile in the eluting solvent was raised from 0 to 28% over 10 min, and from 28 to 42% over 30 min. The absorbance was monitored at 206 nm using a Spectrasystem UV 2000 detector (Thermoquest Limited, Manchester, UK). The percentage of peptide hydrolysed by DPP IV was calculated from dividing the integrated HPLC peak area of the degraded GIP(3–42) fragment by the sum of the peak areas of the intact GIP(1–42) peptide and the GIP(3–42) fragment.

2.5. Acute tests for insulin secretion

BRIN-BD11 cells derived from the electrofusion of New England Deaconess Hospital (NEDH) rat pancreatic β-cells and the immortal rat insulinoma RINm5F cells (McClenaghan et al., 1996) were employed to assess insulin releasing activity. Before experimentation, BRIN-BD11 cells were harvested from the surface of the tissue culture flasks with the aid of trypsin/EDTA (Gibco), seeded into 24-multiwell plates (Nunc, Roskilde, Denmark) at a density of 1.0×10^5 cells per well, and allowed to attach overnight at 37 °C. Acute tests for insulin release were preceded by 40 min pre-incubation at 37 °C in 1.0 ml Krebs Ringer bicarbonate buffer (115 mmol/l NaCl, 4.7 mmol/l KCl, 1.28 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 10 mmol/l $NaHCO_3$, containing 0.5% (w/v) bovine serum albumin, pH 7.4) supplemented with 1.1 mmol/l glucose. Test incubations were performed (n=8) in the presence of 5.6 mmol/l glucose with a range of concentrations (10^{-12} to 10^{-7} mol/l) of GIP and (Ser^2–Asp^{13})GIP (in the absence and presence of 10^{-7} mol/l GIP). After 20 min incubation at 37 °C, samples of the incubation were taken and stored at −20 °C for insulin radioimmunoassay (RIA).

2.6. Analyses

Insulin was determined by dextran-charcoal RIA (Flatt and Bailey, 1981) using guinea pig antiporcine insulin antiserum, rat insulin standard and ^{125}I-labelled

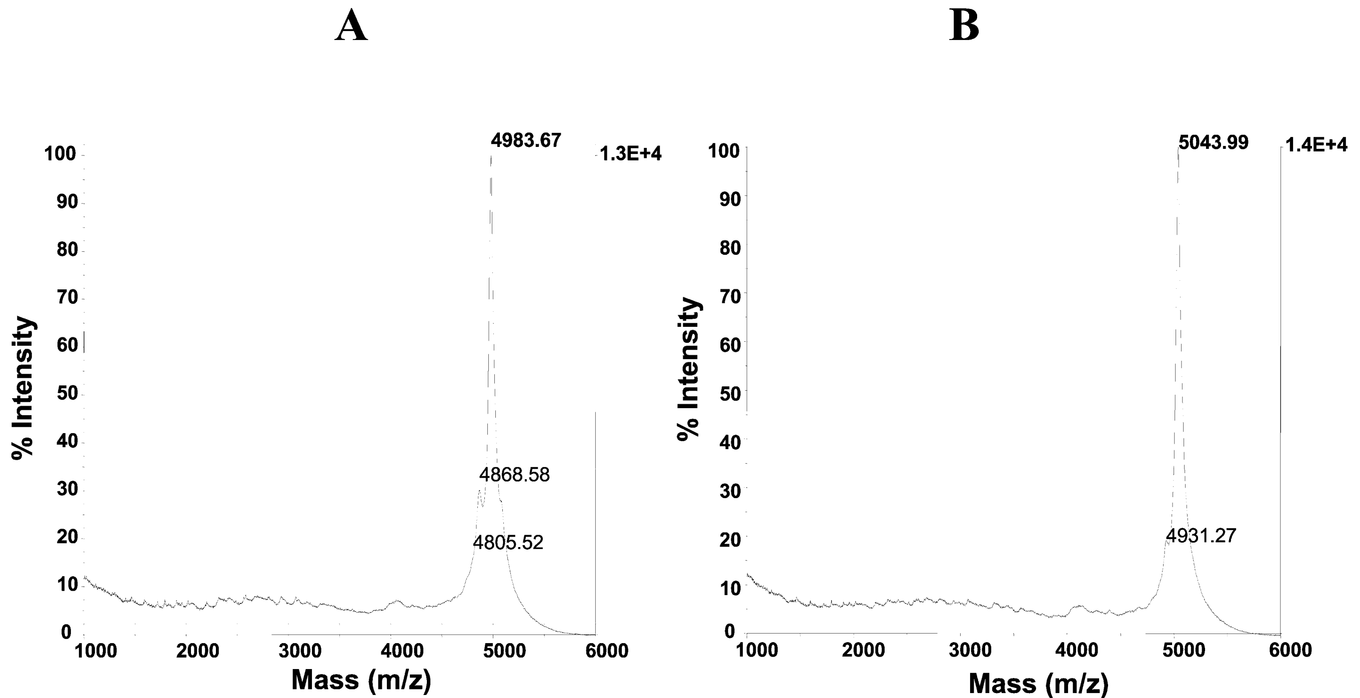

Fig. 1. Matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectra of (A) GIP and (B) (Ser^2–Asp^{13})GIP. The peptides (1 µl) were mixed with 0.5 µl of a matrix solution (10 mg/ml solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/ethanol) and mass spectra recorded using a Voyager-DE BioSpectrometry Workstation. Masses were recorded as mass-to-charge (*m/z*) ratio (ordinate) against relative peak intensity (abscissa).

bovine insulin. Data are expressed as means ± SEM and compared using the Student's *t*-test. Groups of data were considered to be significantly different if $P<0.05$.

3. Results

Following solid-phase peptide synthesis and HPLC purification of GIP and (Ser^2–Asp^{13})GIP, their molecular masses were determined using MALDI-TOF mass spectrometry (Fig. 1). The *m/z* ratio for native GIP was detected at 4983.67 Da, which corresponds closely to the theoretical mass of 4980.5 Da (Fig. 1A). Similarly, for (Ser^2–Asp^{13})GIP the *m/z* ratio was detected at 5043.99 Da (theoretical mass 5040.5 Da; Fig. 1B). Therefore, results from MALDI-TOF mass spectrometry confirm that the correct primary structures for native GIP and its related analogue had been synthesised successfully.

In vitro incubations were performed to evaluate the stability of each peptide to degradation by DPP IV. Data were calculated as a percentage of the intact peptide remaining relative to their respective truncated (3–42) fragment peptide. As shown in Table 1, degradation of native GIP was evident after just 2 h (52 ± 3%), with only 23 ± 3% remaining intact after 4 h. In comparison, (Ser^2–Asp^{13})GIP displayed a moderately reduced hydrolysis rate when incubated with DPP IV, with 65 ± 4% and 59 ± 1% ($P<0.001$) remaining intact after 2 and 4 h, respectively (Table 1). From degradation profiles, the half-live ($t_{1/2}$) of GIP and (Ser^2–Asp^{13})GIP was shown to be 2.3 and >4 h, respectively.

Table 1
Susceptibility of GIP and (Ser^2–Asp^{13})GIP to in vitro degradation by DPP IV

Peptide	% Intact peptide remaining after time (h)			Half-life ($t_{1/2}$; h)
	0	2	4	
GIP	100	52 ± 3	23 ± 1	2.3
(Ser^2–Asp^{13})GIP	100	65 ± 4	59 ± 1***	>4

Data represent the percentage of intact peptide remaining following HPLC separation relative to the major degradation fragment GIP(3–42) after incubation with purified DPP IV. Also included in the Table is the half-life of degradation ($t_{1/2}$). The reactions were performed in triplicate and the mean ± SEM values calculated. ***$P<0.001$ compared with native GIP.

Fig. 2 demonstrates the effects of GIP and (Ser^2–Asp^{13})GIP on insulin secretion from the clonal pancreatic beta-cell line, BRIN-BD11. From Fig. 2A, it can be seen that native GIP dose-dependently (10^{-10} to 10^{-7} mol/l) stimulated insulin secretion (1.1- to 1.9-fold; $P<0.01$ to $P<0.001$) compared with control incubations (5.6 mmol/l glucose alone). In contrast, (Ser^2–Asp^{13})GIP was significantly less potent at stimulating insulin secretion (60–90%; $P<0.05$ to $P<0.001$) compared with native GIP (Fig. 2A). As (Ser^2–Asp^{13})GIP exhibited extremely

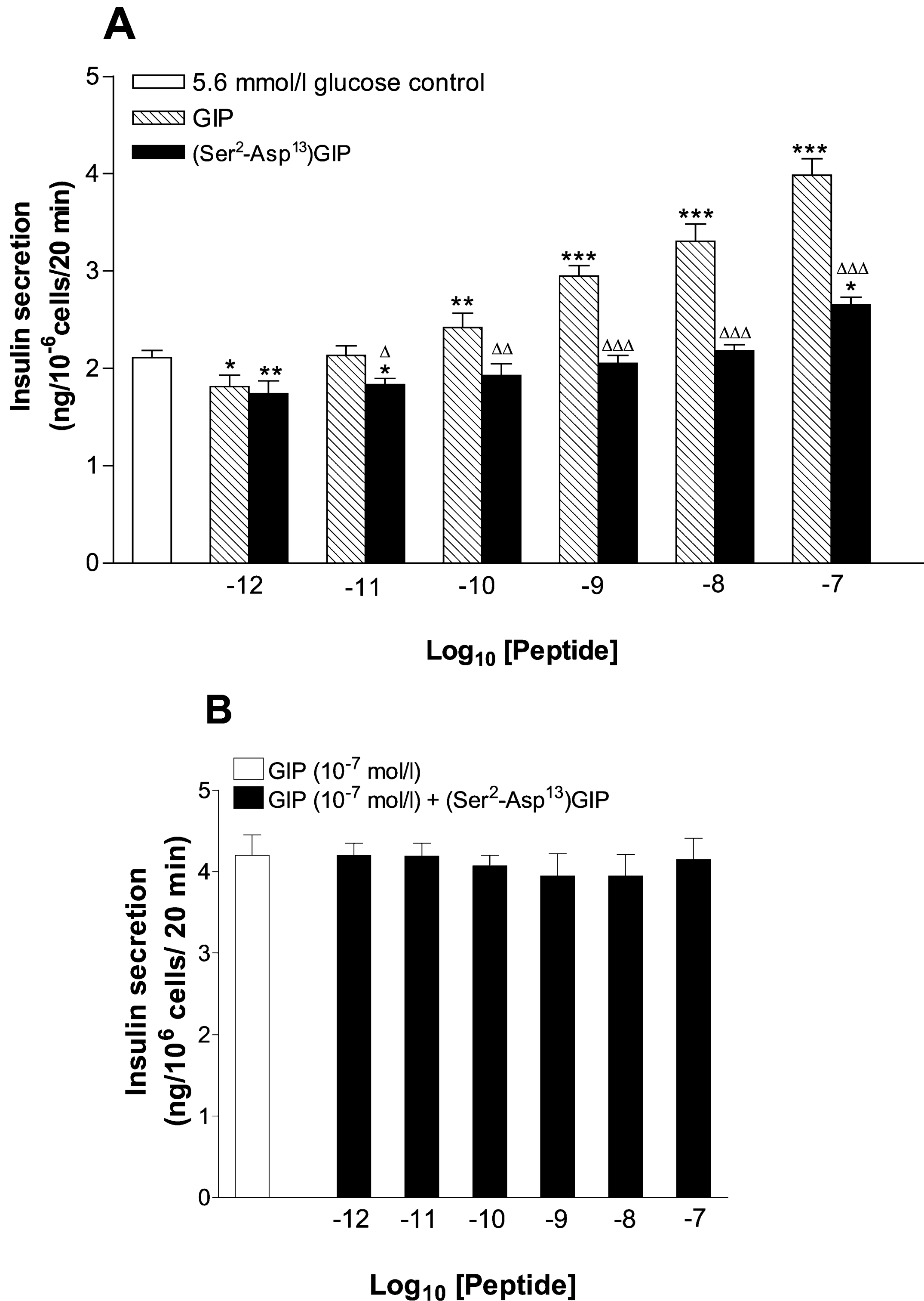

Fig. 2. Effects of GIP and (Ser[2]–Asp[13])GIP on insulin secretion from clonal BRIN-BD11 cells. (A) Dose-dependent effects of GIP and (Ser[2]–Asp[13])GIP. (B) Effects of (Ser[2]–Asp[13])GIP in the presence of 10^{-7} mol/l native GIP. Values represent means ± SEM for eight observations. *P<0.05, **P<0.01, ***P<0.001 compared with control (5.6 mmol/l glucose alone). ΔP<0.05, ΔΔP<0.01, ΔΔΔP<0.001 compared with native GIP at the same concentration.

weak insulinotropic activity, its ability to act as an antagonist of GIP-stimulated insulin secretion was examined (Fig. 2B). However, in the presence of 10^{-7} mol/l native GIP, (Ser[2]–Asp[13])GIP (10^{-12} to 10^{-7} mol/l) had no significant effect on modulating insulin secretion.

4. Discussion

GIP is an incretin hormone present in the epithelial K-cells of the duodenum and jejunum (Buchan et al., 1978). Secretion of GIP is stimulated by digestive products of carbohydrate, fat and protein degradation and is strictly coupled with nutrient absorption (Creutzfeldt, 2001). Its glucose-dependent insulinotropic action provides a self-limiting mechanism, thereby reducing the risk of postprandial hypoglycaemia (Creutzfeldt, 2001), and making GIP attractive as a potential antidiabetic drug.

Although GIP, like GLP-1 (Holst, 1999), might become a therapeutic option in the treatment of type 2 diabetes, one of the most challenging problems in realising its potential antidiabetic properties is its short biological half-life in the circulation (Deacon et al., 2000). GIP has been shown to be degraded in a tissue-specific manner by the ubiquitous enzyme DPP IV (Deacon et al., 2001). The action of DPP IV results in the formation of the truncated GIP(3–42) metabolite which has recently been shown to act as a receptor antagonist against the intact native hormone (Flatt et al., 2001). In order to avoid rapid degradation by DPP IV (half-life 5–7 min) and the unwanted accumulation of antagonist GIP(3–42), the use of DPP IV inhibitors has been proposed. Studies in anaesthetised pigs and laboratory animals have shown that DPP IV inhibitors potentiate the insulinotropic and antihyperglycaemic effects of GIP through prolonging its biological half-life and preventing the build-up of GIP(3–42) antagonist (Deacon et al., 2001; Pospisilik et al., 2002). To date, though, the long-term efficiency and toxicity of such compounds, particularly in man, is unknown and therefore, reservation against this approach seems justified.

One of the more effective and promising alternative strategies to modify GIP action is to develop potent GIP analogues by modification of the primary structure of GIP. This approach has proven very successful in our laboratory, as N-terminally glycated GIP (Tyr^{1}-glucitol GIP) demonstrated noticeably enhanced insulinotropic activity in clonal pancreatic beta-cells (O'Harte et al., 1998), and improved insulinotropic and antihyperglycaemic effects in an animal model of type 2 diabetes (O'Harte et al., 2000). Although N-terminal modifications improve the biological activity of the peptide, other bioactive domains within the molecule have been characterised, the most recent being GIP(1–14) (Hinke et al., 2001). In the same report, computer-assisted analysis predicted the existence of an alpha-helical secondary structure between residues 10 and 29, although preservation of this helical structure in regulating biological activity has not been demonstrated. Therefore, the present study examined a novel di-substituted analogue of GIP, (Ser^{2}–Asp^{13})GIP, to test for DPP IV resistance and the effects of the negatively charged Asp^{13} residue in regulating insulinotropic activity in vitro.

(Ser^{2}–Asp^{13})GIP displayed a more prolonged half-life compared with the native peptide when incubated with DPP IV, consistent with a recent study in our laboratory using (Ser^{2})GIP (Gault et al., 2001). Previous studies have shown GIP to be a potent stimulator of insulin secretion in vitro (O'Harte et al., 1998; Pederson and Brown, 1976). Concordant with this, the current study demonstrated that GIP dose-dependently stimulated insulin secretion in clonal pancreatic BRIN-BD11 cells at 5.6 mmol/l glucose. In contrast, (Ser^{2}–Asp^{13})GIP displayed significantly reduced insulinotropic activity, suggesting that it could act as an antagonist, as has been described for the novel (Pro^{3})GIP analogue (Gault et al., 2002). However, when incubated in the presence of GIP, (Ser^{2}–Asp^{13})GIP failed to influence the biological activity of the native peptide, therefore ruling out possible antagonistic behaviour. The reduced insulinotropic activity of (Ser^{2}–Asp^{13})GIP is in marked contrast to (Ser^{2})GIP, which was more potent in stimulating insulin secretion compared with native GIP (Gault et al., 2001). Thus substitution of Ala^{13} to Asp^{13} has greatly diminished the insulinotropic activity of (Ser^{2}–Asp^{13})GIP. Therefore, the negatively charged Asp residue probably interferes with the secondary structure, due likely as a result of charge-to-charge interactions or destabilisation.

In conclusion, this study has demonstrated that a novel di-substituted analogue of GIP, (Ser^{2}–Asp^{13})GIP, exhibits increased resistance to DPP IV degradation. Novel substitution of Ala in position 13 with a negatively charged Asp residue results in diminished biological activity in vitro. Therefore, we have demonstrated the potential importance of conserving the secondary structure in regulating biological activity.

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