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# Backbone cyclic insulin

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**Backbone cyclic insulin was designed and prepared by reverse proteolysis in partial organic solvent of a single-chain precursor expressed in yeast. The precursor contains two loops to bridge the two chains of native insulin. The cyclisation method uses *Achromobacter lyticus* protease and should be generally applicable to proteins with *C*-terminal lysine and proximal *N*-terminal. The presence of the ring-closing bond and the native insulin disulfide patterns were documented by LC–MS peptide maps. The cyclic insulin was shown to be inert towards degradation by CPY, but was somewhat labile towards chymotrypsin. Intravenous administration of the cyclic insulin to Wistar rats showed the compounds to be equipotent to HI despite much lower insulin receptor affinity. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.**

**Keywords:** insulin; cyclic; semi-synthesis; *Achromobacter lyticus* protease; carboxypeptidase Y; Wistar rats

## Introduction

Backbone cyclic peptides and proteins are of interest due to potential stabilisation towards proteases. Aminopeptidases and carboxypeptidases should be unable to digest proteins with no terminal positions, and stabilisations against endopeptidases are also possible due to a potentially tighter fold of the cyclic structure. Stabilisation of peptide and protein drugs towards proteolytic degradation can be advantageous especially in administrations by aggressive digestive routes such as pulmonary or oral delivery. Although native proteins are in general linear, there are examples of bacterial and snail cyclic peptides as well as many examples of engineered cyclic protein backbones [1–7].

Chemical synthesis allows cyclisation of the terminals of suitably protected peptides or small proteins. Chemical synthesis is however typically limited to sequences less than about 50 amino acid residues and is generally expensive compared to recombinant methods. In addition, the presence of multiple disulfide bridges can complicate synthetic preparations of cyclic peptide structures. Native chemical ligation and intein-based splicing methods have been used for cyclisation of peptide/protein backbones [8–10], but native ligation generally requires an *N*-terminal cysteine and reducing conditions. These requirements can complicate applications to proteins with disulfide bridges.

Amide bonds can be formed by reverse proteolysis in partial organic solvent on unprotected proteins. A range of proteases have been used, although not all proteins tolerate organic solvents. ALP cleaves at the carboxylic side of lysine residues and seems particularly suited and robust in reverse proteolysis. ALP has been exploited for attachment or exchange of segments in among other insulin [11,12]. Insulin contains two chains of 21 and 30 residues, respectively, as well as three disulfide bridges. Reverse proteolysis allows transformation of porcine insulin to HI (by exchange of the *C*-terminal position in the B-chain) as well as preparation of single-chain insulin by forming a bond between the A-chain *N*-terminal and the B-chain *C*-terminal [13].

In healthy humans, insulin is produced in the beta cells of the pancreas as a single-chain precursor, proinsulin [14,15], which contains a 35-residue C-peptide, connecting the *C*-terminal of the B-chain with the *N*-terminal of the A-chain (Figure 1). Proinsulin is considered biologically inactive and the biological activity is only established after maturation into two-chained insulin. For genetic engineering, there has been interest in making biologically active single-chain insulins, because the processing to two-chain insulin is difficult to achieve in recombinant expression systems [16]. It was thus discovered that insulin containing C-peptides of about ten residues can be biologically active [17,18]. Single-chain insulins with short C-peptides have also been shown to be resistant to fibrillation [19].

Inverted proinsulin where the proinsulin C-peptide was moved to the position between the A-chain *C*-terminal and the B-chain *N*-terminal has been engineered (Figure 2) and shown to partially retain receptor affinity and biological activity (contrary to native proinsulin) [20].

We report here the design of a linear precursor containing a short engineered C-peptide, as well as native C-peptide in the 'inverted' loop position (as defined above). The precursor contains

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**Abbreviations used:** *ALP, Achromobacter lyticus protease (alias endo-proteinase Lys-C); CPY, carboxypeptidase Y; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HI, human insulin; NMF, N-methylformamide; NMP, N-methyl-pyrrolidone; V8, V8 protease (alias endoproteinase Glu-C).*



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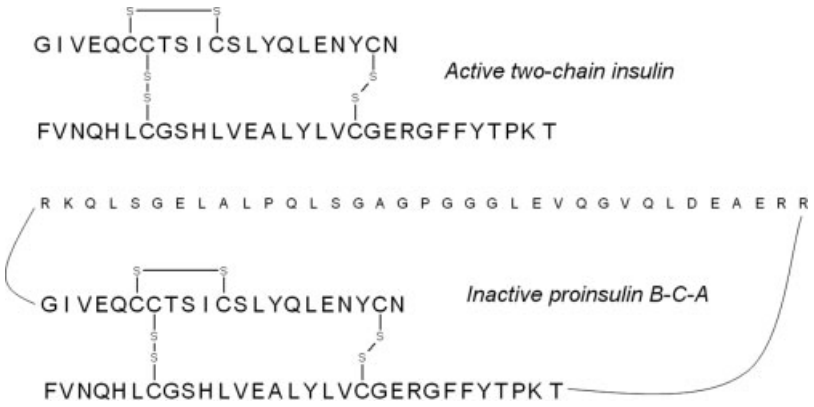


**Figure 1.** Native two-chain insulin (top) and proinsulin (bottom).

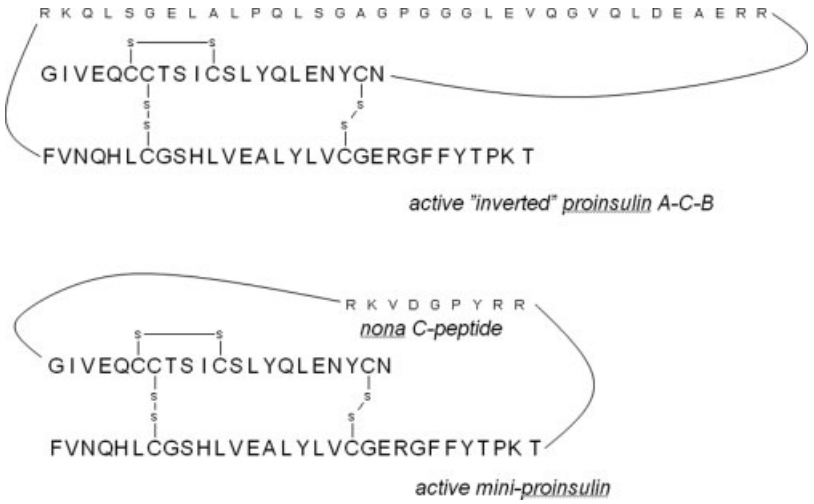


**Figure 2.** 'Inverted' proinsulin (top) and engineered mini-proinsulin (bottom).

lysine in the *C*-terminal position, and by reverse proteolysis with ALP, the precursor can be transformed to cyclic insulin, which is shown to be resistant to cleavage by CPY and equipotent to HI in Wistar rats.

## Materials and Methods

### Recombinant Expression

A synthetic DNA fragment encoding the precursor to the backbone cyclic insulin was obtained from Geneart AG (Regensburg, Germany). The synthetic DNA was subcloned into a cPOT type expression vector previously described in detail [21]. This expression vector has been designed to direct recombinant proteins through the yeast secretory pathway by coupling to the $\alpha$-factor prepro-sequence in the configuration: prepro-peptide-KR-insulin precursor, where KR is a Kex2p protease processing site. The resulting plasmid was transformed into *Saccharomyces cerevisiae* strain MT663. Yeast transformants harbouring the plasmid were selected by glucose utilisation as carbon source on 1% yeast extract, 2% peptone, 2% glucose, agar plates.

### Fermentation, Inoculum Preparation and Growth Conditions

The *S. cerevisiae* strain was inoculated into 5 ml pre-cultures in 50 ml tubes and incubated for 24 h. The pre-cultures were used to inoculate 500 ml baffled shake-flasks containing 150 ml medium. The medium for the inoculum preparation was based on the medium developed by Verduyn *et al*. [22] with the addition of 5 g/l yeast extract and 30 g/l glucose. The flasks were incubated with shaking at 28 °C for 24 h and used to inoculate the fermenters. Aerobic chemostat cultivation was performed at a dilution rate of 0.08 $h^{-1}$ (BIOSTAT® B Sartorius-Stedim, Aubagne, France). The cultivations were performed at 28 °C with a stirrer speed of 1000 rpm and an air flow of 1 vvm. The pH was maintained at 5.9 with ammonia 10% (v/v). The same medium was used as in the inoculum preparation with a glucose concentration of 150 g/l. Silicone antifoam agent (0.5 ml/l) was added to the medium.

### Precursor Purification

The insulin precursor **1** was isolated by capture on a cation exchanger, followed by a purification step on C18 reversed phase HPLC in acetonitrile–water solvents. The product was freeze-dried from this pool.

### Insulin Cyclisation and Purification

The precursor **1** (100 mg, 11 µmol) was dissolved in NMF (20 ml) and water (1.0 ml) and pH was adjusted to 7.2 using 1 M HCl. ALP (Sigma–Aldrich, Brondby, Denmark) stock solution (5.4 mg/ml, pH 7.4, 1.0 ml) was added, and the mixture was left



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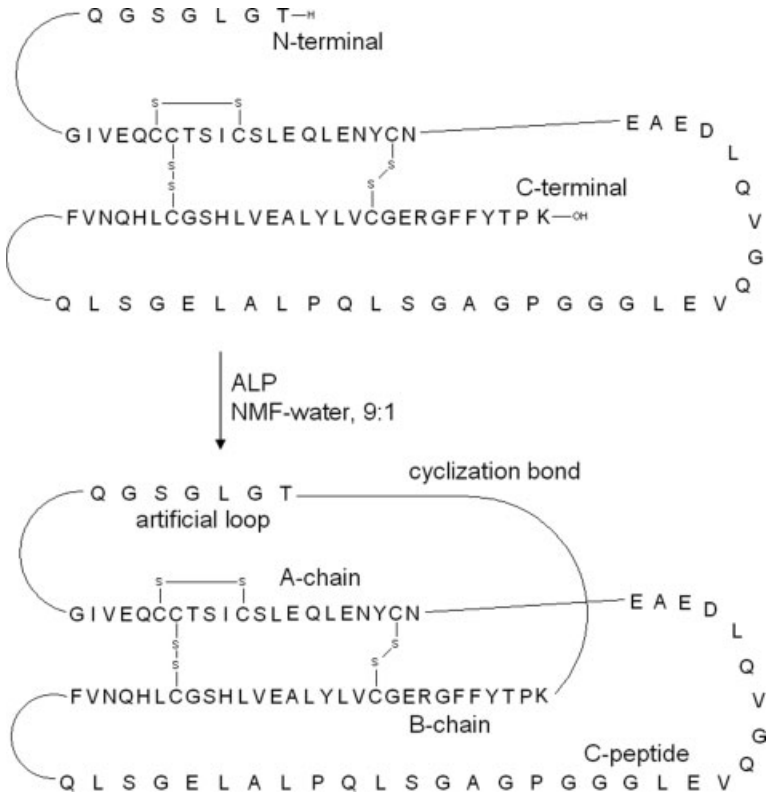


**Figure 3.** Linear precursor **1** and its transformation to backbone cyclic insulin **2**.

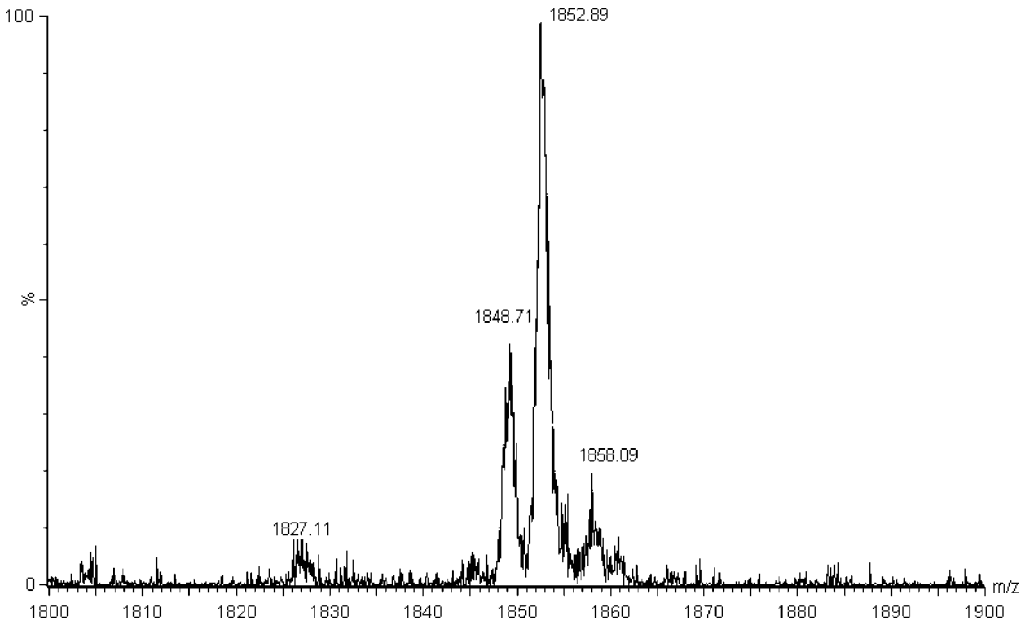


**Figure 4.** Total ion count trace from LC–MS of crude reaction mixture (3.9–4.2 min, 1800–1900 Da) displaying the backbone cyclic insulin product **2** [$M^{5+}$] = 1848.7 Da, linear precursor **1** [$M^{5+}$] = 1852.9 Da and *N*-formyl by-product **3** [$M^{5+}$] = 1858.1 Da.

at room temperature for 4 h. LC–MS showed approximately 50% conversion to the cyclic product **2** (M – water). The crude reaction mixture was diluted with 50 mM ammonium acetate, pH 3.5 (20 ml). The sample was aliquoted into five portions, which were each loaded on Resource S column (GE-Healthcare, Amersham, UK) and eluted with gradient 50 mM ammonium acetate to 30% 2 M ammonium acetate, pH 3.5, over six column volumes, flow 4 ml/min. The collected fraction of peak 1 was pooled and evaporated. The sample was redissolved in water and further purified/desalted by RP-HPLC (C18 column, Daiso 300 Å, 5 µm) using 0.1% TFA in water/0.1% TFA in acetonitrile, 20–55% over 30 min, flow 10 ml/min. The collected fractions were lyophilised to provide backbone cyclic insulin **2** (20 mg, 20%). LC–MS *m/z*: 1848.7 [$M^{5+}$]. Calculated for $C_{402}H_{619}N_{107}O_{131}S_6$: 1848.9 [$M^{5+}$].



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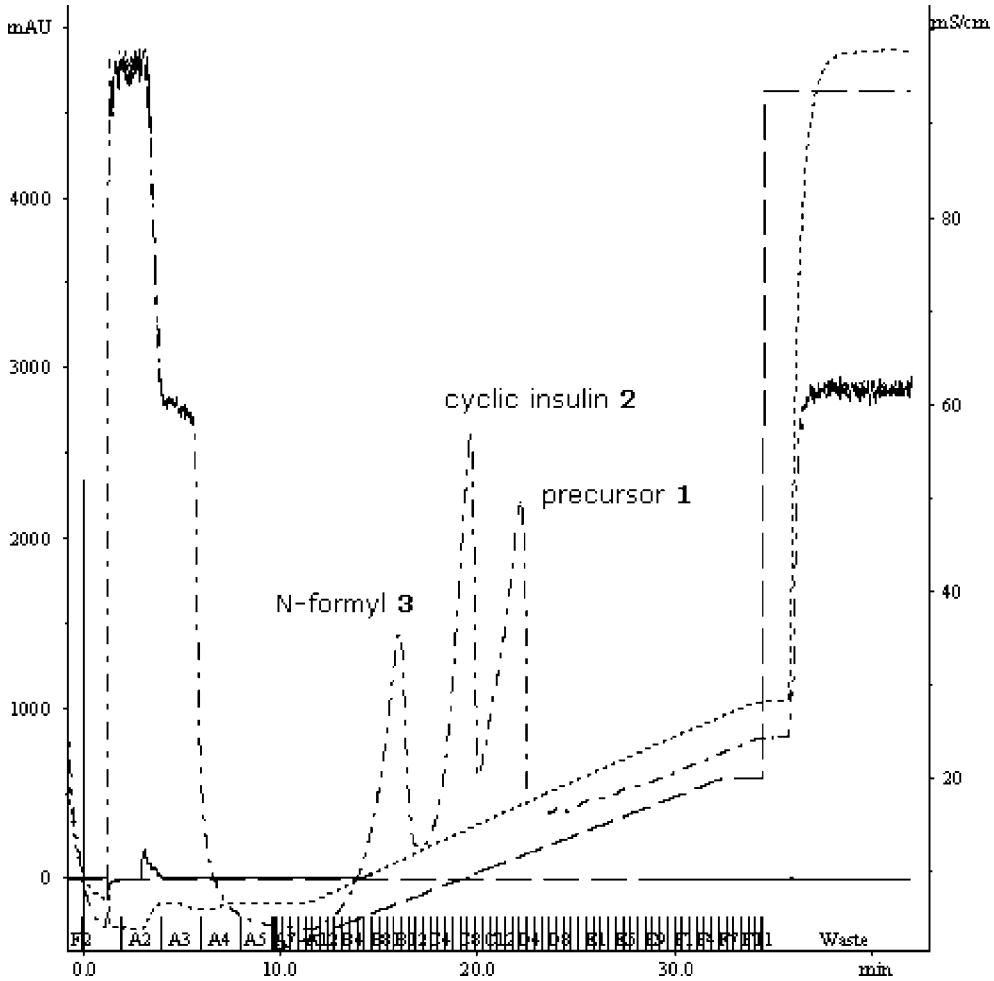


**Figure 5.** Cation exchange-based separation of *N*-formyl by-product **3**, backbone cyclic insulin **2** and linear precursor **1**. Further purification done by RP-HPLC.

### V8 Characterisation

The linear precursor **1** (700 pmol) and the cyclic insulin **2** (700 pmol) were each digested with V8 protease (2 μg, endoproteinase Glu-C, Roche, Indianapolis, IN, USA) in 70 μl of 0.1 M HEPES buffer, pH 7.4 for 16 h at 37 °C. Aliquots (10 μl) were analysed by LC–MS (Ultimate 3000 HPLC) equipped with 1 : 20 flow splitter (Dionex, Sunnyvale, CA, USA) and HCT Ultra PTM ion trap MS system (Bruker Daltonics, Bremen, Germany). RP-HPLC separation was performed using a Zorbax C18 (0.3 × 150 mm) column (Agilent, Santa Clara, CA, USA) with a linear gradient of acetonitrile in 0.1% TFA at 5 μl/min.

### CPY Degradation Study

The linear precursor **1** (600 pmol) and the cyclic insulin **2** (600 pmol) were each incubated with CPY (14 U, Worthington, Lakewood, NJ, USA) in 0.1 M HEPES, pH 7.4 (60 μl) at 37 °C. Aliquots were withdrawn at indicated times, quenched by addition of 10% TFA and analysed by Autoflex II MALDI-TOF MS (Bruker Daltonics, Bremen, Germany).

### Chymotrypsin Degradation Study

The linear precursor **1** (6 nmol) and the cyclic insulin **2** (6 nmol) were each incubated with chymotrypsin (1.7 mg, Sigma–Aldrich) in 0.1 M HEPES, pH 7.4 (200 μl) at 37 °C. Aliquots were withdrawn at indicated times, quenched by addition of 10% TFA and analysed by Autoflex II MALDI-TOF MS and LC–MS as described above.

### *In vivo* Effect of Cyclic Insulin 2 in Wistar Rats

Twenty five male, fed Wistar rats (253–319 g, Taconic, Hudson, NY, USA), were anesthetised using Hypnorm-Dormicum [0.081 mg/ml fentanyl citrate (VetaPharma, Leeds, UK), 1.25 mg/ml Midazolam (Roche, Indianapolis, IN, USA)] 2 ml/kg as a priming dose (to timepoint −35 min) and additional 1 ml/kg to timepoint −5 min prior to test substance dosing, and then 1 ml/kg every 45 min (four times).

The rats were allocated into five groups, five rats in each. The animals were dosed with an intravenous injection in a tail vein (1 ml/kg) of either vehicle (5 mM phosphate buffer, 140 mM NaCl, 70 ppm polysorbate 20, pH 7.4) or cyclic insulin **2** (1.2 or 3.6 nmol/kg) or HI (1.2 or 3.6 nmol/kg). Blood samples for the determination of whole blood glucose concentration were collected in heparinised 10 μl glass tubes by puncture of the capillary vessels in the tail tip to time −15 min and 0 min before dosing, and to time 3, 7, 15, 30, 60, 120, 180 and 240 min after dosing. Blood glucose concentrations were measured after dilution in analysis buffer (500 μl) by the immobilised glucose oxidase



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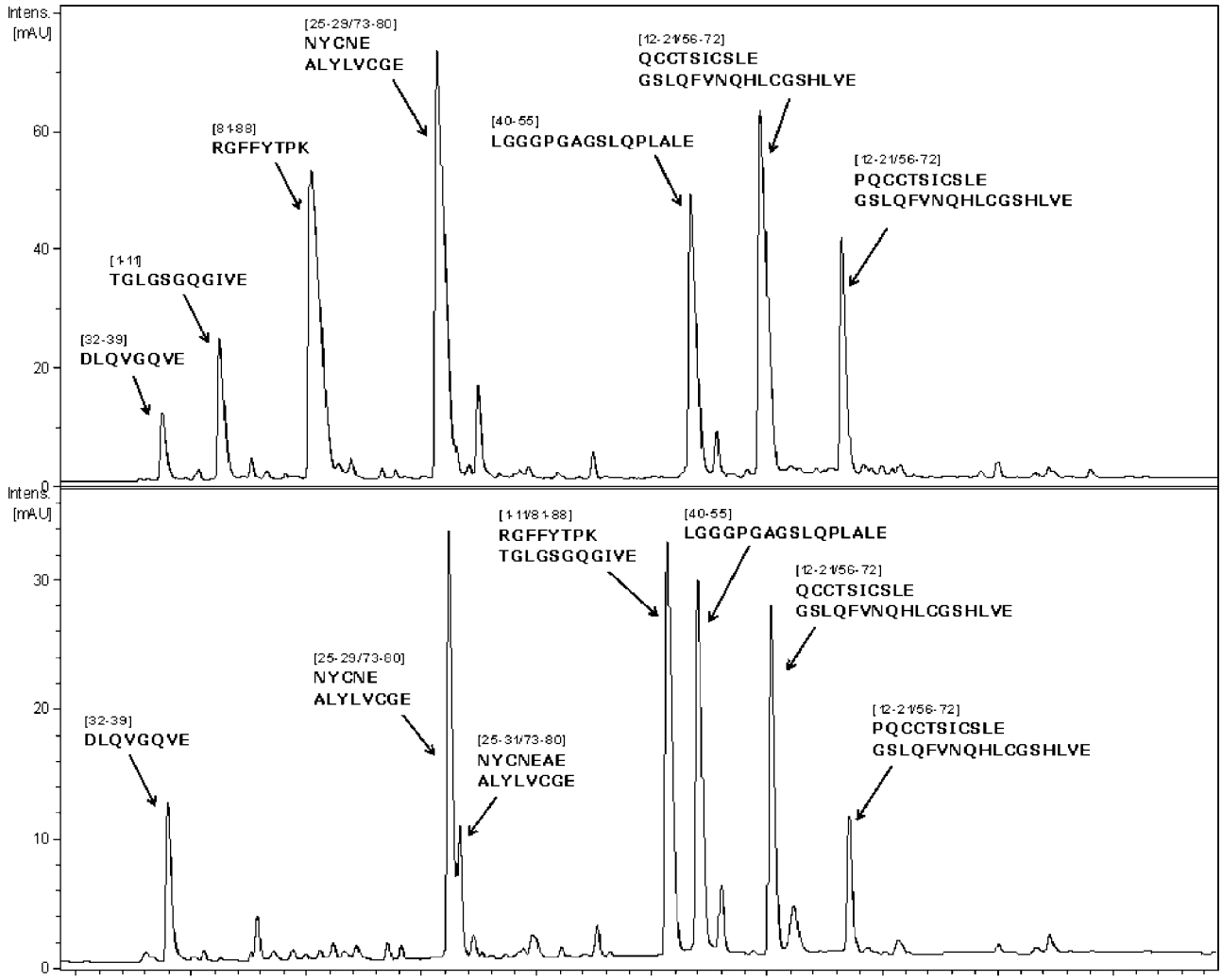


**Figure 6.** Peptide maps of linear precursor **1** (top) and backbone cyclic insulin **2** (bottom) showing the engineered cyclo-bond B29-D1 in fragment RGFFYTP**K-T**GLGSGQGIVE. In addition, the native disulfide patterns are apparent in two fragments observed in both the linear and the backbone cyclic compounds.

method using a Biosen autoanalyzer (EKF Diagnostic, Barleben, Germany).

## Results and Discussion

Single-chain precursors to insulin can be expressed in yeast (e.g. *S. cerevisiae*), which typically secrete folded insulin into the growth medium. This is contrary to *Escherichia coli* expression, where unfolded, insoluble protein in the form of inclusion bodies is often the main product. Figure 3 displays our designed single-chain precursor containing the two extra loops, namely the nonapeptide [18] between the B-chain *C*-terminal and the A-chain *N*-terminal, as well as the native C-peptide in its inverted position [20], between the A-chain *C*-terminal and the B-chain *N*-terminal. The given nonapeptide loop was chosen in the design because it is known to preserve high insulin receptor affinity [18]. To enable the expression of the precursor in yeast, proinsulin C-peptide loop was modified by removing the dibasic sites at both ends (KR and RR). In addition, an A14E mutation [23] was introduced in the construct as this mutation is known to improve insulin expression yields in yeast.

Expression of the precursor for backbone cyclic insulin was obtained using the $\alpha$-leader sequence to facilitate secretion and the Kex2p endoprotease for maturation [24]. An expression plasmid encoding the $\alpha$-leader/insulin precursor fusion was transformed into *S. cerevisiae* and the resulting strain was shown to secrete correctly matured single-chain insulin precursor.

The single-chain insulin precursor **1** was produced by fermentation in continuous culture [22] and isolated by cation column capture followed by purification by RP-HPLC.

For backbone cyclisation, the precursor was treated with ALP in (partial) organic solvents: neat DMF, DMF–water, DMF–ethanol–water, DMSO–water, NMP–water and NMF–water. The following conditions were tested using LC–MS for reaction mixture analysis: insulin concentrations 0.5–10 mM; ALP concentrations 0.1–2% (w/w); pH values 6, 7, 8 or 9; incubations at 5 or 25 °C for up to 3 days and water contents in the range 0–25%. The starting material and the product could not be separated under the acidic LC–MS conditions, but the formation of cyclic product **2** could be monitored in the mass trail as $[M^{5+}]$ (Figure 4). Surprisingly, the reaction worked well only in NMF-based solvent. DMF as co-solvent provided small amounts of the desired product (<5%), whereas in NMP and DMSO only



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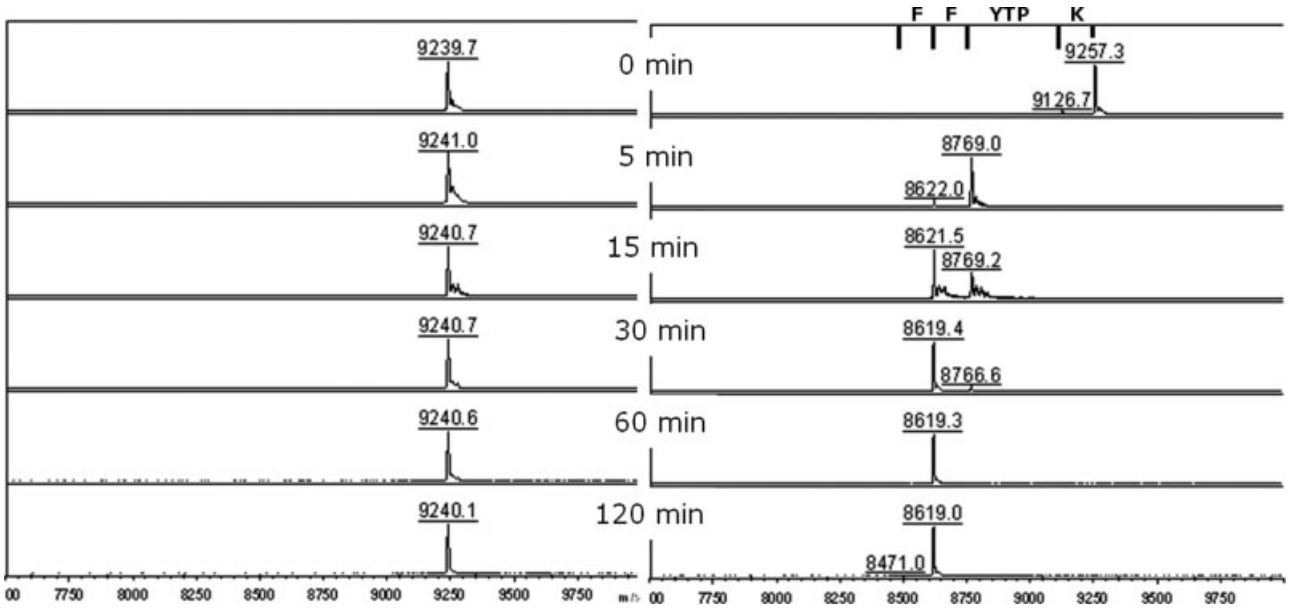


**Figure 7.** MALDI-MS showing the fast digestion of linear precursor **1** by CPY (right) and the stability of backbone cyclic insulin **2** towards CPY (left).

trace of product could be detected. The best identified conditions were NMF–water 20 : 1, 0.5 mM insulin precursor, 2% ALP, pH 7.0, room temperature, providing crude yields of the cyclic product up to 50% after 4 h. The mass analysis from reactions in NMF also showed small amounts of a by-product **3** corresponding to mass of starting material + 29 Da. This corresponds to starting material acylated with formic acid (*N*-formylation). Cation exchange chromatography was used for preparative separation of the desired product **2** from starting material **1** and *N*-formyl by-product **3**. The sample was loaded on sulfonate resin and eluted with a gradient of 50 mM to 1 M ammonium acetate, pH 3, whereby the components where separated as shown in Figure 5. The product was desalted and further purified by RP-HPLC in 0.1% TFA–water–acetonitrile to provide an overall yield of 20% cyclic insulin **2**.

The formation of the ring-closing bond and the presence of native insulin disulfide patterns were documented by digestions using V8 protease followed by LC–MS analysis. V8 protease cleaves peptides after glutamic acid residues. As expected, the precursor **1** therefore digests to give fragments including B22-B29 and C1-A4 (Figure 6). On the other hand, the cyclic product **2** digests with V8 to give only one fragment from this region, B22-A4, 2015.1 Da. This fragment includes the cyclisation bond between B29-lysine and threonine in the *N*-terminal of the nonapeptide (the amino acid sequence of this fragment was confirmed by MS–MS fragmentation, data not shown). Furthermore, the V8 protease peptide mass fingerprints show fragments containing the native disulfides A7-B7 and A20-B19 in both precursor and cyclic product.

The linear precursor and the cyclic insulin were both treated with CPY. The four *C*-terminal residues of the linear sequence **1** were removed within 15 min of incubation (B29-B26), followed by slower processing of the two phenylalanine residues (B25 and B24; Figure 7). CPY was unable to proceed past the arginine–glycine bond (B23-B22), which is known to be resistant to CPY digestion [25]. Gratifyingly, the cyclic compound **2** was resistant to CPY proteolysis over more than 4 h of incubation. Furthermore, the linear precursor **1** was quickly degraded by chymotrypsin (no intact **1** could be detected 5 min after incubation), whereas cyclic compound **2** was still approximately 50% intact 30 min after chymotrypsin incubation.

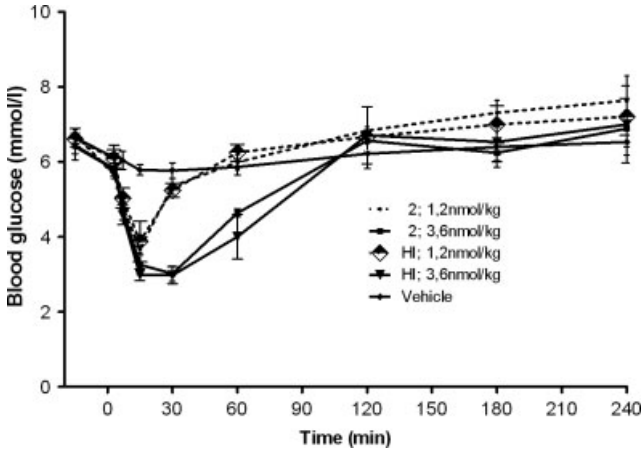


**Figure 8.** Blood glucose profiles following i.v. administration of 1.2 nmol/kg HI (◊), 3.6 nmol/kg HI (▼), 1.2 nmol/kg cyclic insulin **2** (●), 3.6 nmol/kg cyclic insulin **2** (■) and vehicle (♦) in fed anaesthetised Wistar rats, mean values ± standard error of the mean, $n = 5$.

The insulin receptor affinities of the compounds were measured in scintillation proximity assay [26]. $EC_{50}$ was 0.45 nM for the precursor **1** and 1.6 nM for the cyclic insulin **2**. This corresponds to 6 and 1.7% relative to the affinity of HI in the same assay (27 pM). Traditionally, insulins with such low relative receptor affinities would have been considered of low potency or inactive [27], although it is known that insulin analogues can be full agonists despite low receptor affinity relative to native insulin [21].

The cyclic insulin **2** was tested *in vivo* in comparison to HI with two doses administered intravenously to Wistar rats (Figure 8). The effects on blood glucose were similar for the cyclic and the native insulins, which suggest that the cyclic insulin is equipotent to native insulin *in vivo*, despite the much lower receptor affinity. As mentioned earlier, there is precedence for *in vivo* equipotency of insulin analogues to native insulin despite low insulin receptor



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affinity. With glucose utilisation as the measure of insulin *in vivo* potency, the equipotency of low-affinity analogues may be understood by considering that insulin is normally cleared by insulin receptor binding followed by cellular internalisation, which lead to stimulation of cellular glucose uptake. Low insulin receptor affinity of a given insulin analogue can result in clearing by other routes than internalisation becoming significant, such as enzymatic degradation or kidney clearance, but in cases where the receptor-mediated internalisation route is still dominant, the lower insulin receptor affinity may not lead to lower *in vivo* potency. Notably, we cannot exclude the possibility that the flexible loops of the cyclic insulin are cleaved by native proteases, so that the observed effect on blood glucose could be a result of *in vivo* transformation of cyclic insulin to a linear form.

## Conclusion

A linear precursor for backbone cyclic insulin was devised by equipping insulin with two suitable loops. The precursor was expressed and secreted from yeast as folded single-chain insulin. ALP treatment in 95% aqueous NMF provided up to 50% transformation to backbone cyclic insulin. Curiously, the cyclisation proceeded well only in aqueous NMF and not in other solvents. *N*-formylated insulin was observed as a by-product when using NMF, but the by-product could be removed by ion exchange chromatography. Backbone cyclic insulin was shown to be inert towards proteolysis by CPY and was degraded by chymotrypsin slower than the linear precursor. The cyclic insulin showed significantly decreased insulin receptor affinity relative to HI, but the compound nevertheless appeared equipotent to HI *in vivo* as studied by intravenous administration to Wistar rats.

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