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Preparation and Analysis of Acylated Insulin with Dehydrocholic Acid

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Abstract: Insulin was chemically modified with dehydrocholic acid without the use of protecting agents and the main monoacylated insulin. ε-N^{B29}-Dehydrocholyl insulin was obtained selectively and analyzed by PAGE, HPLC and MALDI-TOF-MS.

Keywords: Insulin, dehydrocholic acid, dehydrocholyl insulin, chemical modification.

Insulin is the most important regulatory hormone in the control of glucose homeostasis and generally administered by injections in the treatment of diabetes mellitus. So far, many approaches, such as various dosage forms, protease inhibitors and absorption enhancers, have been attempted to overcome the disadvantage of insulin delivery[1]. Chemical modification of insulin may be a potential useful approach to improve the selective absorption of insulin, protect it from degradation by intestinal proteases and prolong the duration of action in the body[2-5]. There are three primary amino groups on insulin available for chemical modification, namely the α-amino groups of Gly^{A1} and Phe^{B1}, respectively, and the ε-amino group of Lys^{B29}. The modification of these sites may lead to a mixture of mono-, di- and even tri-modified products. The modification at Phe^{B1} or Lys^{B29} did not affect the biological activity of insulin, while the modification at Gly^{A1} stimulated the decrease in activity[6]. Especially, the modification of Lys^{B29} residue may minimize trypsinic cleavage because it is one of the main target sites of trypsin. However, the site-specific modification invariably required the use of specific protecting agents[7,8], which involved a multiple-step reaction, separation and purification.

In this work, a novel acylated insulin, ε-N^{B29}-dehydrocholyl insulin **4** was prepared by covalent linkage of dehydrocholic acid (DHC) **2** to porcine insulin without protecting agents and analyzed by polyacrylamide gel electrophoresis (PAGE), reversed phase high performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Experimental

The approach to preparing **4** was based on the synthesis of 1-dehydrocholyl benzotriazole

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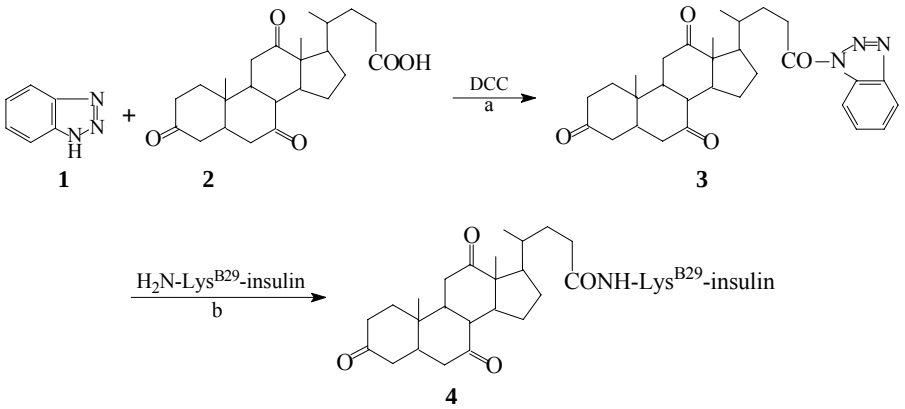
Scheme 1



1 **2** **3** **4**

Reagents and conditions: a) anhydrous ether, 0°C; b) pH>10, 0°C.

3, an activated amide. Benzotriazole **1** and **2** were dissolved in anhydrous acetone and then Dicyclohexylcarbodiimide (DCC) in anhydrous acetone was added at 0 °C. The resulting precipitate was removed and the filtrate was kept cooling overnight at 0 °C to provide **3**. Insulin was dissolved at 0 °C in the mixture of N-methyl-2-pyrrolidone, water and triethylamine (TEA), keeping pH over 10. Subsequently, a solution of **3** in N-methyl-2-pyrrolidone was added and stirred for 200 min. Then acetone was added. The resulting suspension was isolated by centrifugation (-10°C, 10000 rpm). The supernatant was removed and the residue was then lyophilized to provide **4** (**Scheme 1**).

PAGE analysis of native insulin and the modification product were performed on a Hoefer miniVE vertical electrophoresis system by a discontinuous polyacrylamide gel with a running gel and a stacking gel, which consisted of 20% and 5% acrylamide respectively. The gels were stained with Coomassie brilliant blue.

The crude synthetic product was analyzed by RP-HPLC on HP Agilent 1100 with an autoinjector using a Jupiter C18 reversed phase column (5 μm, 250 mm ×4.6 mm, 300 Å, Phenomenex Co.) and UV detection at 214 nm. Gradient elution was performed. The mobile phase was a mixture of 0.1 mol/L pH 3.0 phosphate buffer and acetonitrile. The flow rate was 1 mL/min and the column temperature was 30 °C.

The mass spectrometric analysis of the modification product was conducted on a Bruker BIFLEX^{III} time-of-flight mass spectrometer (Bruker Daltonics, Germany). CHCA was used as a matrix. All mass spectra shown were acquired in the positive ion mode and the given molecular weight represented masses of the compounds.

Results and Discussion

3 was obtained in a yield of 60% directly from **1** and **2** in one step by DCC coupling method. The activated amide **3** was reacted with unprotected insulin and showed a selective acylation of one of the three free amino groups on insulin in the mixture of water and N-methyl-2-pyrrolidone at pH above 10 in the presence of TEA. Generally, the acylation of ε-amino group of Lys^{B29} was dominant at pH over 10 because of a

relatively higher pKa value of Lys^{B29} (11.2). As a result, the major acylated insulin was **4**, ε-N^{B29}-dehydrocholyl insulin, yet a little amount of unreacted insulin remained.

The modified insulin and unreacted insulin can be simply separated by PAGE (**Figure 1**). Comparing to that of native insulin (Lane 2), a distinctly faster band corresponding to new species was resolved for the crude modification product (Lane 1). This faster migrating band appears to reflect at least a kind of modified insulin. Nevertheless, PAGE failed to adequately identify each component of the modification mixture. In other words, the faster migrating band should be composed of several modification products.

RP-HPLC analysis showed that the retention time of native insulin was 9.2 min. There were four well-resolved elution peaks A, B, C and D for the modification product with retention times of 9.2, 17.4, 20.2 and 23.1 min respectively (**Figure 2**). The single peaks at different positions showed isolated compounds. Considering the increase of the lipophilicity of insulin after chemical modification with DHC, the retention times of the modified insulin were longer than that of native insulin. In comparison with that of the native insulin, the tentative identities of peaks A, B, C and D were assigned as the unreacted, mono-, di- and tri-dehydrocholyl insulin, respectively. In addition, the major product was purified by RP-HPLC with a semi-prep Hypersil C18 reversed phase column. The purified monoacylated insulin was reconstituted and analyzed once again by RP-HPLC and showed a purity of above 95%, no di- or tri-acylated product was observed (the **Figure 2** not shown).

Figure 1 Polyacrylamide gel electrophoresis using discontinuous gels for samples.

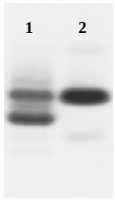
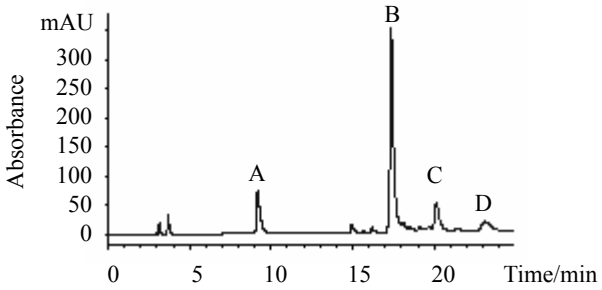
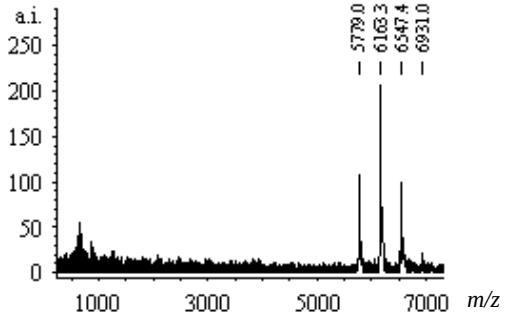


Figure 2 RP-HPLC of the modification products by C18 reversed phase column.



MALDI-TOF-MS of the modification product displayed three typical mass signals at *m/z* 5779, 6163, 6547 Da and a minor peak at *m/z* 6931 Da, separating from each other by 384 Da and corresponding to the mass of adduct with H^+ of the native insulin, mono-, di- and tri-dehydrocholyl insulin, respectively (**Figure 3**). The strongest mass peak, 6163.3, which is 384 Da heavier than control insulin, is ascribed to that of **4** and reveals that it was successful in selective acylation of the unprotected insulin at the ε-amino group of Lys^{B29} residue with **3**. The components and masses of the modification product were directly identified by MALDI-TOF-MS. Furthermore, the mass of the purified monoacylated insulin was confirmed by the same method (figure not shown). In addition, MALDI-TOF analysis of the fragments of the modified insulin treated by DTT and specific enzyme confirmed that the main site of modification was ε-amino group of Lys^{B29} (data and figure not shown). In other words, the major product was **4**. Animal experiments showed that **4** retained glucose-lowering effect, extended the action time as well as a protective effect from trypsinic degradation.

Figure 3 MALDI-TOF-MS of the modification product



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