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Full Length Research Paper

Characterization of recombinant Arabian camel (*Camelus dromedarius*) insulin

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The production of hormones by biotechnological approaches has contributed significantly to treatment of many diseases. DNA recombinant technology has facilitated production of new forms of insulin from many species and mammalian insulin to be used as a therapy for diabetic patients. In this study, proinsulin from Arabian camel was produced and characterized for the first time by recombinant technology. Recombinant camel proinsulin was cloned and expressed in *Escherichia coli* to be produced and characterized it *in vitro*. Camel proinsulin sequence was compared with human insulin sequences. Camel proinsulin is 5.8 kDa in size and includes 87 amino acids with highly conserved domains. Proinsulins are highly conserved enzymes in many mammals; camel proinsulin possesses 87.5% homology to human proinsulin by amino acid sequences. The C peptide chain is made up of a total of 35 amino acids of which 27 amino acids are identical and the remaining are variable. The three dimensional structure of camel proinsulin was deduced for molecule homology studies with human proinsulin. The results suggest that, camel proinsulin cDNA may be used as a specific probe for proinsulin studies with other organisms and may serve biotechnology field as a model for future comparative enzymatic, therapeutic and pharmaceutical studies.

Key words: Camel, cloning, insulin, human, alignments, sequencing, 3D-structure.

INTRODUCTION

Recombinant DNA technology in the recent years has revolutionized the sector of biopharmaceutical industries. Insulin homologs were developed and approved for clinical human use. Proinsulin is the precursor prohormone to insulin synthesized in β cell of the islets of Langerhans. After the synthesis, it is processed by some proteases to form mature insulin. The protein is encoded by a single gene in most species. The insulin gene is located on chromosome 11 in humans (Owerbach et al., 1980) and composed of two intervening sequences. The first sequence is located in the 5' part of the mRNA and the second resides in the C-peptide region of proinsulin (Bell et al., 1980). Human insulin includes two separate chains: A-chain (21 amino acids) and the B-chain (30 amino acids) joined by a characteristic pattern of disulfide bridges. These chains are contained within a single polypeptide chain and are processed by proteolytic processing to proinsulin and eventually to insulin. The two chains are connected by a portion known as the C-peptide, which is removed before insulin is secreted but is released together with the insulin (Chang et al., 1998). Nearly all animals make and use insulin as a hormonal control of metabolism (Floyd et al., 1999). Human insulin is manufactured by molecular biology techniques for clinical use either in *Escherichia coli* or yeast (Chan et al., 1981; Chance et al., 1981; Thim et al., 1986). Although, the presence of insulin gene in camels had long been inferred, the specific gene had never been identified. The chemical structure of a large number of insulin molecules from mammals was determined (Smith 1972) including native (cellular) camel insulin which was crystallized and characterized in 1972 (Danhu, 1972). Until now, no reliable work has been done to isolate the insulin gene from camel. Many camels in Saudi Arabia are suffering

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from fetal diseases, which may have an affect on the economy of the country. Accordingly, the big demand for camel insulin production is sought for treatments. In this study we aimed to produce active recombinant camel proinsulin in *E. coli* and characterize the sequences of Saudi Arabian camels (*Camelus dromedarius*) to use it subsequently in many down stream biotechnological and disease treatment applications.

MATERIALS AND METHODS

Sample collection and RNA extraction

All tissue and blood camel samples were collected from a local commercial slaughterhouse in Riyadh. Pancreatic tissue samples were collected from freshly sacrificed adults. The whole pancreas organs were removed as quickly as possible (approximately 15~25 min post scarification), weighted and stored on ice. Several pancreatic tissue samples were excised from several locations, quickly cut into slices less than 0.5 cm in thickness and immediately submerged into 2 ml sterile centrifuge tubes containing RNAlater® RNA Stabilization Reagent (Qiagen) prior to RNA extraction process. Total RNAs were extracted from pancreatic tissue samples by using QuickPrep total RNA extraction kit (Amersham Biosciences) according to the manufacturer's protocol. Pre-weighted (~100 mg) of pancreatic tissues were removed from RNAlater® RNA stabilization reagent and extraction buffers and immediately submerged into 1.5 ml eppendorf tube containing 225 µl β-mercaptoethanol. Samples were homogenized thoroughly on ice by using tissue grinder (Biospec products, INC) at highest speed. Lithium chloride solution (525 µl) was added and homogenization continued to obtain a homogeneous suspension. CsTFA (750 µl) were added to samples, vortexed thoroughly and stored on ice for 10 min. Samples were centrifuged at high speed for 15 min at room temperature and supernatants were dispensed carefully by vacuum aspiration. The RNA pellets were washed with 113, 263 and 375 µl of combined extraction buffer, lithium chloride solution and CsTFA, respectively. Samples were centrifuged at high speed for 5 min and supernatants were aspirated without disrupting pellets. The tubes were incubated on ice and washed with 1.5 ml ice-cold 70% ethanol. Samples were centrifuged at high speed and the ethanol supernatants were discarded carefully. RNA pellets were dissolved in 100~200 µl of DEPC-treated water and incubated on ice for 15 min. To further ensure that the pellet is dissolved, RNA pellets were incubated at 65ºC and repeatedly mixed by pipetting or pulse vortexing. The yield and purity of RNA were assessed by spectrophotometric analysis (Nanodrop1000) and the integrity of total RNA extraction was evaluated in MOPS denaturing agarose electrophoresis.

Reverse transcription and cDNA amplification

First-strand cDNA synthesis of camel proinsulin cDNA synthesis was performed by using Ready to go RT-PCR beads kit according to the manufacturer's instruction (Amersham Biosciences). Two sets of reactions were prepared, one utilizing pd (N) 6 random first-strand primer and the other utilizing pd (T) 12-18 first-strand primer. Each reaction volume was 50 µl and contained 23 µl DEPC-treated water, 2.5 mg pd(N)6 random primer or 0.5 mg pd(T) 12-18 primer, 20 pmol human proinsulin gene-specific primer (Cam-INS-F2 = 5`-TTT GTG AAC CAA CAC CTG TGC GGC TC-3`) and 2 mg total RNA. Positive and negative controls were included in each run. Thermocycling conditions were 42ºC for 20 min, 95ºC for 5 min followed by 35 cycles of denaturing at 94ºC/30 s and annealing at 55ºC/45 s and extension at 72ºC/45 s. The final extension cycle was at 72ºC for 5 min. The PCR amplicons were analyzed by 1.5% agarose gel electrophoresis and successfully amplified PCR reactions were used as templates for subsequent gene-specific PCR amplifications. The forward and reverse primers used to amplify camel proinsulin cDNA were designed based on previously published sequences of proinsulin cDNAs of other species, e. g. *Homo sapiens* (Accession NM-000207). Cam-INS-F2 (5`-TTT GTG AAC CAA CAC CTG TGC GGC TC-3`) and Cam-INS-R2 (5`-CGT CTA GTT GCA GTA GTT CTC CAG CTG-3`) were utilized to amplify camel proinsulin cDNA (NT 73 to NT330 corresponding to codon 25 to codon 111). The amplified camel proinsulin cDNA harbors "B" chain, "C' chain and "A" chain, respectively. The PCR reaction volume was 50 µl and contained 2 µl of first-strand PCR reaction, 200 µM dNTPs, 400 nM of appropriate primers, 0.2 µl of 5 Units/µl, Taq DNA polymerase (Amersham Biosciences) and 5 µl of 10X PCR buffer. A Flexigene PCR system was used to run all PCR reactions. The cycling conditions were: 40 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 45 s and polymerization at 72°C for 45 s, followed by final extension at 72°C for 5 min. The PCR products were analyzed on 1.5% agarose gel and a 264 bp discrete band was confirmed. As a positive control for successful RT-PCR and first-strand cDNA synthesis, Globin gene-specific primers Glbn-F (5`-ACA CTT CTG GTC CAG TCC GAC TGA G -3`) and Glbn-R (5`-GCC ACT CAC TCA GAC TTT ATT CAA A -3`) were utilized. The thermocycling and PCR reaction conditions were the same as for camel proinsulin cDNA amplification reaction.

Molecular cloning of camel proinsulin cDNA

A TOPO-TA one-step cloning strategy (Invitrogen) was used for the direct insertion of amplified camel proinsulin cDNA into a linearized plasmid vector according to manufacturer's instructions. Briefly, the camel proinsulin cDNA fragment was sub-cloned in frame into N-terminal 6xHis tagged pCRT7/NT-TOPO vector to create pCI-FS construct. In a microcentrifuge tube, 2 µl of fresh PCR product was mixed with 1 µl salt solution and the total volume was brought up to 5 µl by ddH2O. One µl of linearized pCRT7/NT-TOPO® vector was added and the reaction mix was incubated at room temperature for 5 min. The reaction mix was incubated on ice and 1 µl was used directly to transform competent One Shot TOP10F′ *E. coli* BL21 (Invitrogen) as described in the protocol. The transformed cells were plated on LB plates containing 100 mg/ml ampicillin. The plates were incubated at 37ºC overnight to allow colony growth. Bacterial colonies were screened by growing 10~20 single colonies in 6 ml of LB media containing 100 mg/ml ampicillin for 6~8 h. Bacteria from one ml of LB culture was harvested by centrifuging at 5,000 rpm for 10 min and the bacterial pellet was resuspended in 100 ml ice-cold PBS. The correct orientation of the pCI-FS construct was screened by primer–specific PCR, enzymatic digestion, and direct dideoxy sequencing at KCAST core facility. After confirmation, the pCI-FS construct was transferred to the lysogenic expression host *E. coli* BL21 (DE3) in order to express camel proinsulin.

Expression and purification of camel proinsulin

Expression of recombinant camel proinsulin (pCI-FS) was induced by addition of 1 mM iso-propyl-β-thio-galactopyranoside (IPTG) for 4 h at 30°C in 55.5 mM glucose in Luria-Bertani (LB) medium. Induction was followed by lysis with the BugBuster extraction reagent per manufacturer's instructions (Novagen). Subsequently, lysates were resolved by 10% SDS-PAGE followed by Coomassie Blue and silver staining (Invitrogen). Hexahistidyl-tagged camel proinsulin was bound to Ni-NTA cations in a His-bind resin (Novagen) and eluted with imidazole (1 M). Protein purity was assessed by SDS-PAGE. The protein was dialyzed against PBS in

(A)
TTTTGTGAACCAACACCTGTGCGGCTCACACCTGGTGGAGGCGCTGT
ACCTGGTGTGCGGGGAGCGCGGCTTCTTCTACACGCCCAAGGCCCG
CCGGGAGGTGGAGGACACTCAGGTGGGCGGCGTGGAGCTGGGTGG
AGGCCCGGGTGCGGGCGGCCTGCAGCCCCTGGGCCCGGAGGGGCG
CCCGCAGAAGCGCGGCATCGTGGAGCAGTGCTGCGCCAGCGTCTGC
TCGCTCTACCAGCTGGAGAACTACTGCAACTAGACG

(B)
FVNQHLCGSHLVEALYLVCGERGFFYTPKARREVEDTQVGGVELGGG
PGAGGLQPLGPEGRPQKRGIVEQCCASVCSLYQLENYCN-T

Figure 1. Delineation of camel insulin cDNA and amino acid sequences. (A). The cDNA sequences are composed of 265 base pair. (B) Protein sequences are composed of 87 amino acids.

a cassette Dialyzer (Slid-A- lyzer, Pierce, Rockford, IL) and concentrated by column-facilitated centrifugation (Ultrafree 0.5 µM filter unit, Millipore).

RESULTS

Using degenerate and nested primers, the complete camel proinsulin gene was isolated, cloned, sequenced and characterized (Figure 1). Recombinant camel proinsulin was expressed and purified and yielded a molecular weight of 5.8 kDa when the hexahistidyl is considered (850 Da) (Figure 2). The nucleotide and amino acid sequences (Figures 3 and 4) were determined for the camel proinsulin and the differences found for the corresponding segments of human are shown in Figure 4 (Lasergene version 7, DNAstar). The clones contain 265 bp and the nucleotides are different in 36 positions. The nucleotide sequences diverge at positions (39, 42, 45, 51, 63, 66, 81, 88, 135, 138, 144, 150, 154, 166, 170, 173, 185, 195, 201, 207, 210, 223 and 231 for one bp; at 101, 181 and 216 for two bp; at 109 for three bp and at 120 for four bp). Comparison of amino acid sequences indicates that there are eleven different amino acids in camel proinsulin as compared to that of human. According to their characteristics, they include one polar (threonine), nine non-polar (two alanine, two valine, three glycine and two proline) and one charged (arginine) in camel as compared to five polar (two threonine, one glutamine and two serine) and six non-polar (two alanine, three leucine and one isoleucine) in human. The base substitutions that do occur at positions upstream to 88 bp fail to alter the amino acid sequence of the protein. These characteristic base substitutions and structural features may represent sites of interspecies dissimilarity. In comparison to human proinsulin gene and protein, the camel gene's structure and function were confirmed and found to be 86.36 and 87.5% similar in form and product, respectively, to that of human proinsulin. The C chain has 27/35 identical amino acids (77.14%) and 8 of the amino acids are conserved changes. The camel proinsulin gene has similar three exon (one untranslated), two intron distribution found in all proinsulin genes sequenced to date. It was reported (Danhu, 1972). that, the amino acid

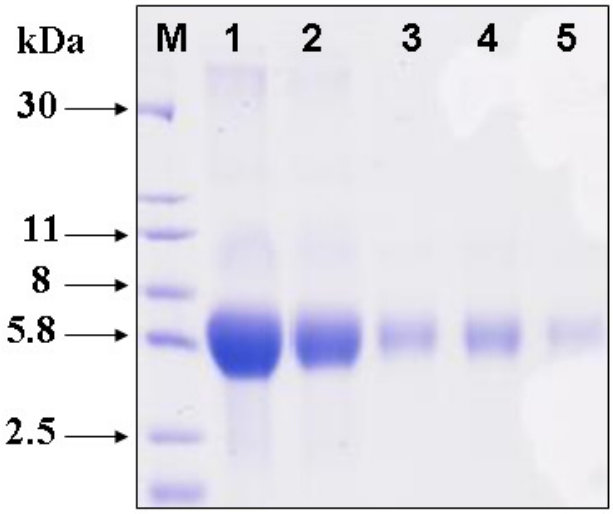

Figure 2. Camel insulin gene was expressed and fractionated by SDS-PAGE before coomassie blue staining and yielded a predominant protein of the expected size (5.8 kDa). Recombinant hexahistidyl-tagged camel insulin was purified by binding to Ni-NTA cations in a His-bind resin (Novagen) and eluted with 1 M imidazole. The protein was dialyzed against PBS to remove denaturing agents, concentrated and stored at -80C in multiple aliquots subsequently. Lanes 1-5 are purified eluate fractions of recombinant camel insulin.

composition of the camel proinsulin (tentative sequence) was identical to that of bovine proinsulin, A chain, where as B chain differed from bovine B chain in that it contained alanine instead of valine in the second position. In the present study, the amino acid sequence of the A and B chains correspond 100% to that of human proinsulin (A and B chains has 21/21 and 30/30 identical amino acids, respectively). Using protein comparative modeling approach, camel proinsulin three dimensional structure was predicted based on the three dimensional structure of human proinsulin (Figure 5) (Guex and Peitsch, 1997; Peitsch et al., 1995; Schwede et al., 2003; Hooft et al., 1996).

DISCUSSION

In this study, cloning and characterizing the first Arabian camel proinsulin were done by recombinant DNA technology. It is composed of 265 bp which encodes 87 amino acids which were found to be 86.36 and 87.5% similar in form, product, and three dimensional structures to human proinsulin at the nucleic acids and amino acids level, respectively. The C peptide chain is made up of a total of 35 amino acids of which 27 amino acids are identical and the remaining 8 amino acids are variable.

The complete amino acid sequence of the insulin molecule was described in the early 1950s; insulin was the first protein to be sequenced entirely (Sanger 1952, Sanger and Thompson, 1953a, b). This pioneering work

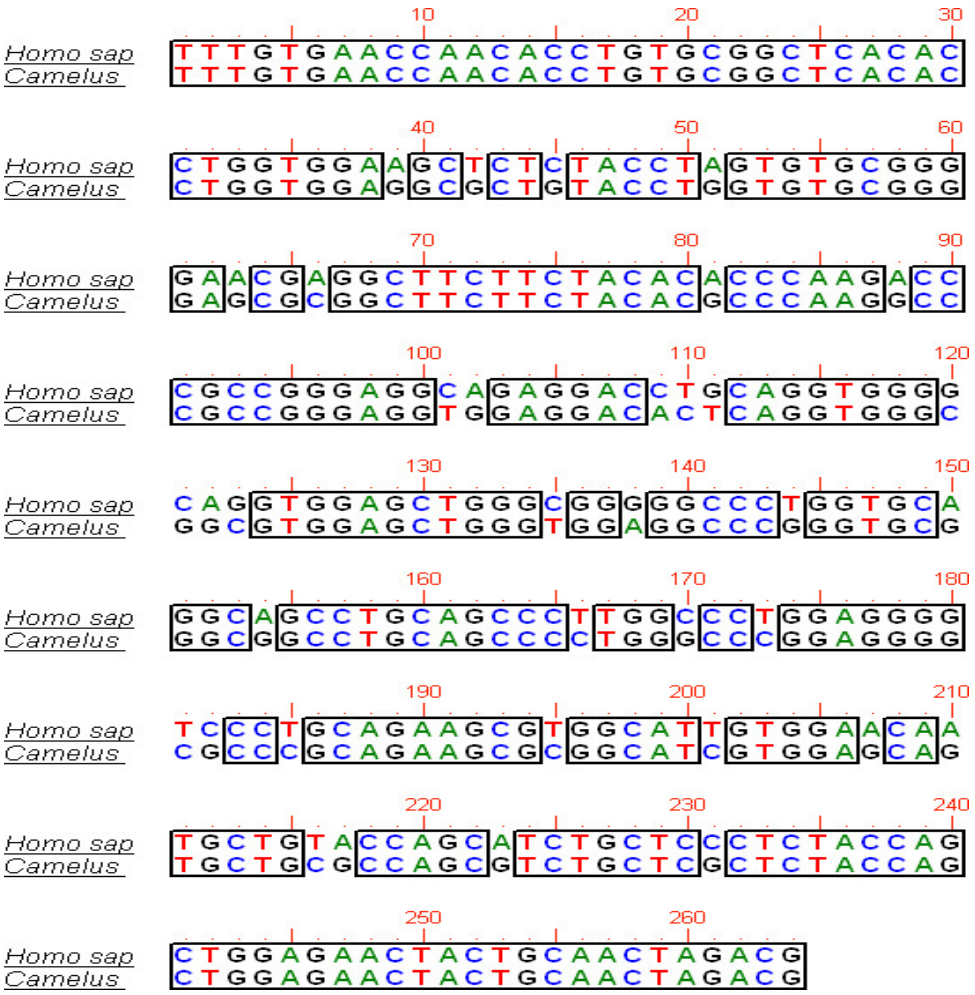

Figure 3. Nucleotide sequences of camel proinsulin (265 bp) were compared with nucleotide sequences of human proinsulin by using Lasergene program (DNAstar, version 5.06). Pair wise alignment of *Homo sapiens* proinsulin cDNA vs. *Camelus dromedarius* proinsulin cDNA sequences: Sequence 1: *Homo sapiens* proinsulin cDNA. Sequence 2: *Camelus dromedarius* proinsulin cDNA. Optimal Global Alignment Homology: 86.36%.

was confirmed from 1963 to 1966, when several groups reported laboratory synthesis of biologically active insulin. According to the published camel insulin sequence, our results indicated that camel proinsulin A chain has 29/30 identical amino acids to the published camel proinsulin sequence, one alanine (A) in the published sequence was replaced by valine (V). These results are important in informing comparative gene prediction. If orthologs between human and camel have virtually identical intron–exon structures, then cases of ambiguous assignment of intron boundaries should be resolvable by comparison with the other species. Reportedly (Steiner et al., 1985) in many species, the A-chain is mainly conserved. Most of the sequences have a few mutations and differences in the amino acid sequence, but over all the A-chain is conserved, while the B-chain and the preproinsulin section is changed. Most of the divergence seems to occur in the B-chain. Mainly the B-chain is omitted from the sequence.

The preproinsulin, amino end has some conservation. The first amino acids tend to change some, but the overall sequence is similar. The proinsulin section also stays intact through out the species above. Therefore, conservation is kept on the carboxyl end of the protein and on the amino end, and diverges in the middle. Consequently, it seems that since preproinsulin is spliced off in early stages of insulin function, it has no reason to adapt to the given species, whereas the proinsulin and B-chain have more of an individual effect on the given protein and species and are more subject to change. It is all part of evolution and if it were not for these natural occurrences, the necessary insulin protein would not be working properly in each species.

Although the amino acid sequence of insulin varies

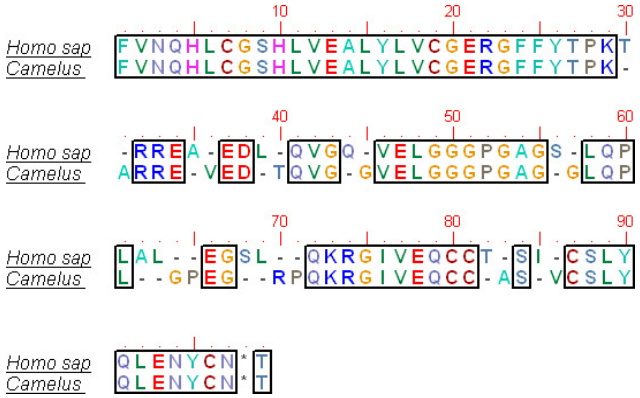

Figure 4. Deduced amino acid sequences of camel proinsulin (87 amino acids). Amino acid sequences of human and camel proinsulins were compared by Lasergene program (DNAstar, version 5.06). Pair wise Alignment of *Homo sapiens* proinsulin vs. *Camelus dromedarius* proinsulin peptide sequences. Sequence 1: *Homo sapiens* proinsulin amino acids. Sequence 2: *Camelus dromedarius* proinsulin amino acids. Optimal Global Alignment Homology: 87.5%.

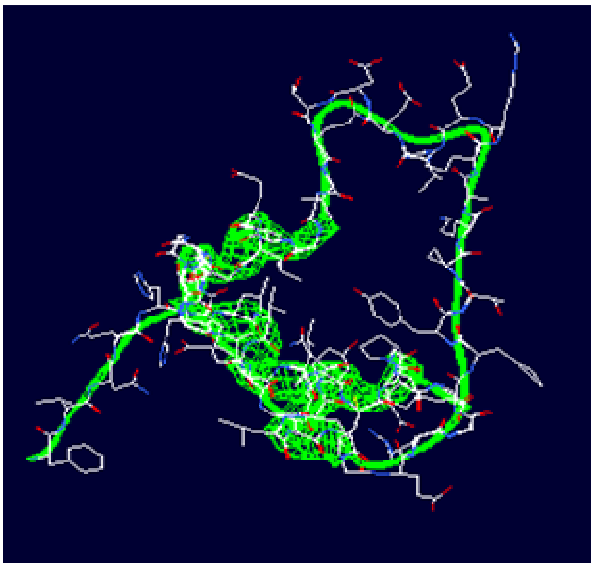

Figure 5.Three dimensional structural prediction of camel insulin (target) was generated based on the three-dimensional structure of human insulin (template) which has high homology levels to the target. The three-dimensional model of camel insulin was constructed by submitting the primary amino acid sequences (target) to the Swiss-model databases (http://swissmodel.expasy.org//SWISS-MODEL.html). The Human insulin was used as a template for alignment with the target protein. The accuracy of the three-dimensional model of camel insulin has been evaluated by the WhatCheck program in the Swiss-model suite. The program generates Z-scores for each criterion used in the evaluation. The Z-scores are defined as the standard deviations from the mean of the expected value. The green line (the predicted structure) is wrapped along the structural template. The general color scheme is as follows: positively charged amino acid residues in blue, negatively charged residues in red and neutral residues in white.

among species, certain segments of the molecule are highly conserved, including the positions of the three disulphide bonds, both ends of the A chain and the C-terminal residues of the B chain (Steiner et al., 1990). In addition to human insulin, insulin protein from different species such as rabbit, sheep, pig, dog, horse, elephant and camel has been studied closely. Despite the species diversity, insulin from all investigated animals harbors a close homology to human insulin. The amino acid differences give each species' insulin a slightly different structure and activity because the whole insulin protein folds around on itself and has very specific locations where it interacts with the insulin receptor on the cell. Because of this very specific structure-activity relationship, a substitution of one amino acid in a critical location may make the protein deform or not work as well in another species. For instance, camel proinsulin has 92.2% homology to human proinsulin. The only differences are at codons 2, 30, 38 and 40. These similarities in the amino acid sequence of proinsulin lead to a three-dimensional conformation of insulin that is very similar among species and insulin from one animal is very likely biologically active in other species. Indeed, pig insulin has been widely used to treat human patients. Looking at the enzyme in more detail, the sequence of porcine (pig) insulin and human insulin is almost identical, but not exactly – it differs by one amino acid. Bovine (beef) insulin is different by three amino acids from human. It is of interest to point out that the newly developed insulin analogs like Lispro, Aspart and Glargine; also differ from 'human insulin' in two or more amino acids. Theoretically changes in amino acid could affect the solubility and diffusion properties of insulin molecules (Jacobs, 1985). Clinical efficacy of insulin clearly does not depend on the species of insulin used. A number of clinical trials have clearly shown that animal insulins and human insulins are comparable in their clinical efficacy. It is also claimed that the doses of human insulin required were also less compared to animal insulin, but this was only true when the animal insulins were impure. The duration of action of human insulin is slightly shorter than animal insulins (Mohan, 2002).

Comparison of the amino acid sequences of our camel proinsulin B chain with those from other species reveals that pig, dog, horse, sheep and ox exhibit no sequence variation (100% homology); man, CEmacaque, rabbit, elephant, chihamster and opossum exhibit no significant variation (96.60%); squirmonk, mouse and rat 1 exhibit very high significant variation (66.60%). On the other hand, A chain exhibits no sequence variation (100%) with ox, no significant variation (95.23%) with sheep, and significant variation (90.47%) with man, CEmacaque, rabbit, pig, dog and elephant. It has high significant variation (85.71%) with horse, rat 1, chihamster and opossum; and very high significant variation with squirmonk (76.19%) and guineapig (57.14%). This difference affords a good criterion to study molecular mechanisms respon-

sible for insulin expression and function in these animal species. These structural differences between species may reflect ancient divergence between alleles on chromosomes indicates a common ancestor between alleles and is informative on different timescales of population history (John and Alec, 2002). Insulin expression profiles can reflect and show characteristic biochemical differences between species. The present results indicate that, cloned camel proinsulin cDNA provides a specific probe for the analysis and isolation of genomic DNA fragments containing proinsulin genes. Further and detailed studies may have important implications and would provide a framework to disease association and gene analysis within diverse populations.

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