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Full-length cDNA cloning and structural characterization of preproinsulin in *Alligator sinensis*

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ABSTRACT. Insulin is an important endocrine hormone that plays a critical physiological role in regulating metabolism and glucostasis in vertebrates. In this study, the complete cDNA of *Alligator sinensis* preproinsulin gene was cloned for the first time by reverse transcription-polymerase chain reaction and rapid amplification of cDNA ends methods; the amino acid sequence encoded and protein structure were analyzed. The full-length of preproinsulin cDNA sequence consists of 528 base pairs (bp), comprising a 34-bp 5'-untranslated region, a 170-bp 3'-untranslated region and an open reading frame that is 324 bp in length. The open reading frame encodes a 107-amino acid preproinsulin with a molecular weight of approximately 12,153.8 Da, theoretical isoelectric point of 5.68, aliphatic index of 92.06, and grand average of hydropathicity of -0.157, from which a signal peptide, a B-chain, a C-peptide, and an A-chain are derived. Online analysis suggested that the deduced preproinsulin amino acid sequence contains a transmembrane region, and that it has a signal peptide whose cleavage site occurs between alanine 24 and alanine 25. Comparative analysis of preproinsulin amino acid sequences indicated that the A-chain and B-chain sequences of

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preproinsulins are highly conserved between reptiles and birds, and that the preproinsulin amino acid sequence of *Alligator sinensis* shares 89% similarity to that of *Chelonia mydas*, but low similarity of 48-63% to those of mammals and fishes. The phylogenetic tree constructed using the neighbor-joining method revealed that preproinsulin of *Alligator sinensis* had high homology with reptiles and birds, such as *Chelonia mydas*, *Gallus gallus*, and *Columba livia*.

Key words: *Alligator sinensis*; cDNA cloning; Homology analysis; Insulin

INTRODUCTION

Insulin is a peptide hormone secreted by B cells of the pancreatic islets of Langerhans (Pessin et al., 2000). It consists of 2 polypeptide chains, the A- and B- chains, linked together by disulfide bonds. Insulin is first synthesized as the single polypeptide preproinsulin. Preproinsulin contains a signal peptide that directs the nascent polypeptide chain to the rough endoplasmic reticulum (RER). The signal peptide is cleaved as the polypeptide is translocated into lumen of the rough endoplasmic reticulum, forming proinsulin. Proinsulin undergoes maturation into active insulin by releasing a fragment known as the C-peptide (Bendzko et al., 1982). Insulin plays an important role in maintaining glucose homeostasis, stimulating glucose and amino acid uptake, and increasing protein synthesis (Montserrat et al., 2007; Enes et al., 2010). Because of its important role in metabolism, insulin hyposecretion or limited insulin cell metabolism can lead to diabetes (Peila et al., 2002).

Insulin is one of the most extensively studied regulatory peptides in vertebrates; structural studies have focused largely on mammals, birds, and teleost fishes (Conlon, 2001; Caruso et al., 2008). To date, although the cDNA sequences for the preproinsulin gene from more than 100 species of vertebrates are listed in GenBank (through October 31, 2013 at NCBI), only 2 were from amphibians or reptiles, including *Xenopus laevis* and *Chelonia mydas*, respectively. No complete sequence of preproinsulin cDNA from crocodilians has been reported.

The Chinese alligator *Alligator sinensis*, which belongs to Crocodilia, Testudinidae, is an endemic species in China and was listed by the Chinese government as a first-level state-protected species in 1972 (Yan et al., 2005). Nature reserves and artificial farms of *Alligator sinensis* were set up in the Anhui and Zhejiang Provinces. In this study, the complete cDNA sequence of the *Alligator sinensis* preproinsulin gene was cloned by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) methods, and its encoding amino acid sequence as well as the protein structure were analyzed. This study could enhance the understanding of preproinsulin gene phylogenetic evolution and provide fundamental data for further studies on the regulation of metabolism in *Alligator sinensis*.

MATERIAL AND METHODS

Total RNA extraction

Adult *Alligator sinensis*, more than 10 years of age, were collected from the Xu-

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anzhou Alligator Culturing Centre in Anhui Province. Pancreatic tissues were excised and immediately kept in RNA-Be-Locker A (Sangon Biotec, Shanghai, China) and then stored in a -80°C refrigerator. Total RNA was extracted from pancreas tissue by using Total RNA Extractor (Sangon Biotec) and quickly grinding the pancreas tissue in liquid nitrogen. The remaining steps were conducted according to manufacturer instructions. Total RNA was stored in a -80°C refrigerator.

cDNA synthesis and RACE

The RNA was reverse transcribed into cDNA using PrimeScript 1st cDNA Synthesis Kit (TaKaRa, Shiga, Japan). The conserved sequence of preproinsulin cDNA of *Gallus gallus*, *Columba livia*, and *Xenopus laevis* were obtained from the GenBank of NCBI to design corresponding primers (Table 1). Total cDNA from the pancreas of *Alligator sinensis* was used as template for subsequent PCR and the partial preproinsulin cDNA were obtained using the primer pair IF1 and IR1. Another primer pair was designed based on the partial insulin cDNA for RACE (Table 1). The primers 5'-GSP1, 5'-GSP2, and 5'-GSP3 were used to obtain the 5' RACE product, while 3'-GSP1 and 3'-GSP2 were used to obtain the 3' RACE product.

Table 1. Primers for preproinsulin cDNA amplification.

Primer name	Primer sequences
IF1	5'-GCTCTCTACCTRGTGTGT-3'
IR1	5'-CTAGTTGCAGTAGTTCTCCAG-3'
5'-GSP1	5'-CCCTCTTCACTTTCTC-3'
5'-GSP2	5'-TCCACTTCGTTCCGCAGAGG-3'
5'-GSP3	5'-AGGCTGTTCAAGGTCCCGTC-3'
3'-GSP1	5'-GACGGGACCTTGAACAGCCTCTAGTG-3'
3'-GSP2	5'-GCCATAACACCTGCTCCCTTTACCAG-3'

All primers used in this study were designed using the Primer Premier 5.0 and synthesized by Sangon Biotec. The 5' RACE reaction was performed using the System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. The 3' RACE reaction was performed using SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to manufacturer instructions.

PCR products were purified using a DNA Gel Extraction Kit (Axygen, Hangzhou, China). Next, purification products were sub-cloned into PMD-18T (Takara) and the recombinant DNA was transformed into DH5α *Escherichia coli* cells (TransGen, Beijing, China). Approximately 9-10 positive clones were sequenced by Sangon Biotech.

Sequence analysis

DNA sequences were searched in the NCBI, aligned using ClustalX, and integrated in the DNASTAR Lasergene software package. The amino acid sequence was deduced using ExPASy (http://web.expasy.org/translate/). The open reading frame ORF was identified using the ORF finder on the NCBI website (http://www.ncbi.nlm.nih.gov./projects/gorf/). Physical parameters of amino acid sequences were determined using the Protparam tool (Wilkins et

al., 1999) (http://web.expasy.org/protparam/). A signal peptide was predicted by the SignalP 4.1 server (Bendtsen et al., 2004) (http://www.cbs.dtu.dk/services/SignalP/). Amino acid sequence transmembrane regions were predicted using the TMpred server (Sun et al., 2009) (http://www.ch.embnet.org/software/TMPRED-form.html). Multiple amino acid sequences of different species were aligned using the ClustalX (Thompson et al., 1994). A phylogenetic tree was constructed using the Mega 5.0. Secondary protein structure was predicted using Jpred 3 (http://www.compbio.dundee.ac.uk/ www-jpred/).

RESULTS

Amplification of full-length preproinsulin cDNA

RNA gel electrophoresis showed that the RNA extraction products had 2 distinct bands of 18s and 28s, which were examined in follow-up experiments. Total RNA was reverse transcribed to synthesize cDNA, which was used as a template for the PCR reactions. After PCR amplification using primer pair IF1 and IR1 (Table 1), 1.5% agarose gel electrophoresis showed that there was a distinct band as the anticipated fragment (Figure 1a). A DNA fragment of 213 base pairs (bp) was obtained.

The primers 5'-GSP1, 5'-GSP2, and 5'-GSP3 (Table 1) were used to obtain the 5' RACE product (Figure 1b). A DNA fragment of 257 bp was obtained after sequencing. The primers 3'-GSP1 and 3'-GSP2 (Table 1) were used to obtain the 3' RACE product (Figure 1c). A DNA fragment of 264 bp was obtained after sequencing. The results of blast analysis using NCBI indicated that the fragments obtained were partial fragments of preproinsulin cDNA.

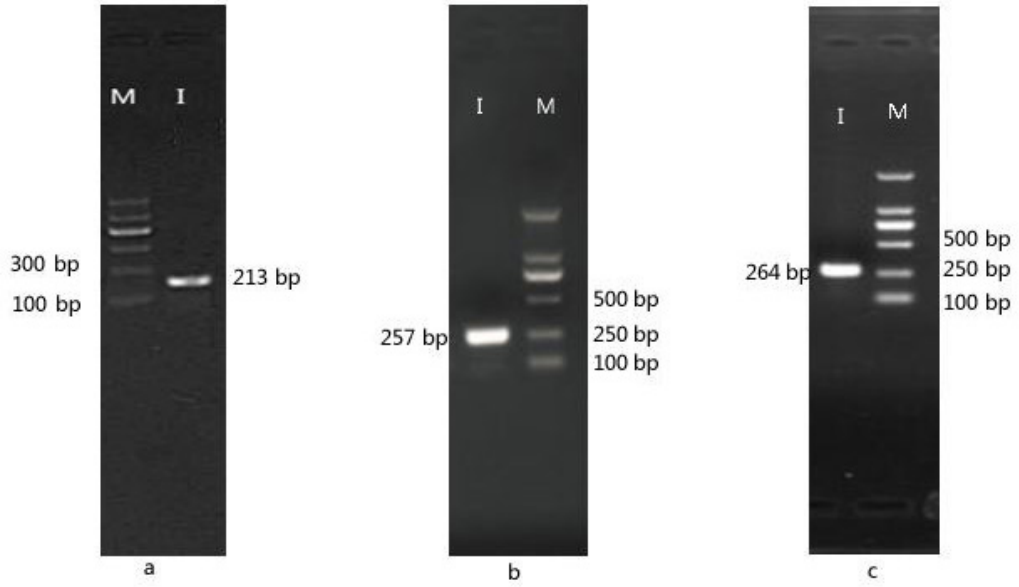

Figure 1. Agarose gel electrophoresis of PCR products. **a.** ordinary PCR product, **b.** 5' RACE product, **c.** 3' RACE product, *lane M* = DNA marker, *lane I* = objective band.

cDNA sequencing and identification

The 5' RACE fragment and the 3' RACE fragment described above were spliced using the DNASTAR Software. The overlapping 5' and 3' RACE fragments yielded a sequence of 528 bp from *Alligator sinensis* (Figure 2). Sequence analyses showed that the fragment contained the complete coding sequence of the preproinsulin cDNA (324 bp), encoding 107 amino acids, including the coding region for the signal peptide (72 bp), the insulin B-chain (90 bp), C-peptide (84 bp), and A-chain (63 bp). The initiation codon and termination codon were ATG and TAG, respectively. The 5' untranslated region (UTR) was 34 bp. The 3' UTR was 170 bp and contained a single typical polyadenylation signal (AATAAA) and poly-A tail (Figure 2).

Figure 2. Full-length cDNA and amino acid sequences of *Alligator sinensis* preproinsulin. ATG shows initiation codon. Upper sequence indicates the amino acids and the lower shows the nucleotide. TAG shows termination codon.

Preproinsulin amino acid sequence analysis

The amino acid sequence was deduced based on the nucleotide sequence. The transmembrane prediction program (TMpred) identified 1 statistically significant potential transmembrane helix at Ser17-Ser33. The signal peptide was analyzed using the SignalP 4.1 server, which showed that the signal peptide cleavage site was between alanine 24 and alanine 25. For the insulin A chain, only 1 cleavage site was located at Ala87-Gly88. For the insulin B-chain, 2 cleavage sites were located at Ala24-Ala25 and Gly54-Arg55.

Sequence analysis showed that preproinsulin from *Alligator sinensis* consisted of a signal peptide with a length of 24 amino acids, a B-chain of 30 amino acids, and a C-peptide with 28 amino acids, and ending with an A-chain that is 21 amino acids long. The molecular weight of the protein was found to be 12,153.8 Da and the formula was $C_{540}H_{836}N_{148}S_7$. Theoretical pI was 5.86 and the instability index (II) was found to be 52.31, a value that classifies the protein as unstable. The aliphatic index, grand average of hydropathicity, and amino acid variation were 92.06, 0.157, and 20, respectively. The abundance of leucine, glutamic acid, alanine, and serine were relatively higher than that of other amino acids, accounting for 15, 8.4, 7.5, and 7.5% of the total amino acids, while threonine and tryptophan relatively lower, accounting for 0.9% (Figure 3).

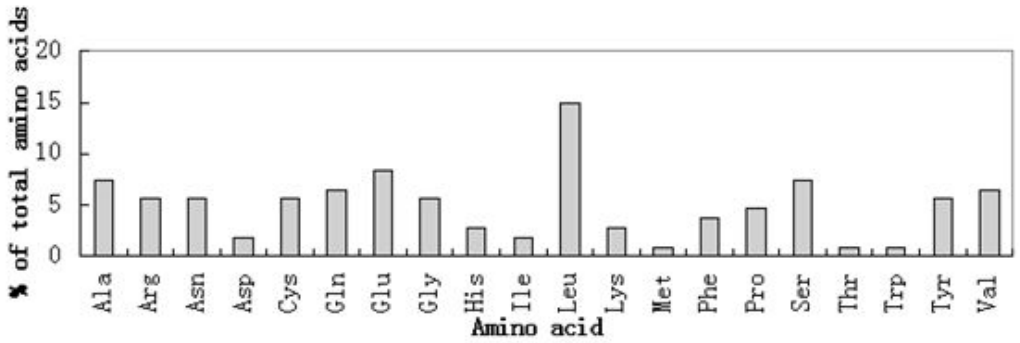

Figure 3. Amino acid composition of preproinsulin from *Alligator sinensis*.

The amino acid sequence comparison of the *Alligator sinensis* and other species, including mammals, birds, reptiles, amphibians, and fishes, indicated high similarity regarding disulfide bond position. Disulfide linkages form between the 2 cysteines in the A-chain (C97-C102) and B-chain (C31-C98, C43-C111) (Figure 4). The N-terminal A-chain of *Alligator sinensis* (A1-A8, GIVEGCCH) was very similar to other species, except *Cavia porcellus* at residue A4 (D), *Homo sapiens*, *Sus scrofa*, and *Cavia porcellus* at residue A8 (T), and *Bos taurus* and *Felis catus* at residue A8 (A). The N-terminal B-chain of *Alligator sinensis* (B1-B6, AANQRL) was similar to that of birds and reptiles (B1-B6, AANQHL). However, this sequence differed from that of mammals, amphibians, and fishes. Additional non-cysteine conserved residues included leucine at B6, B11, and B15, glycine at B7 and B23, serine at B9; valine at B12 and B18, tyrosine at B16 and B26, phenylalanine at B24 and B25, proline at B28 and lysine at B29.

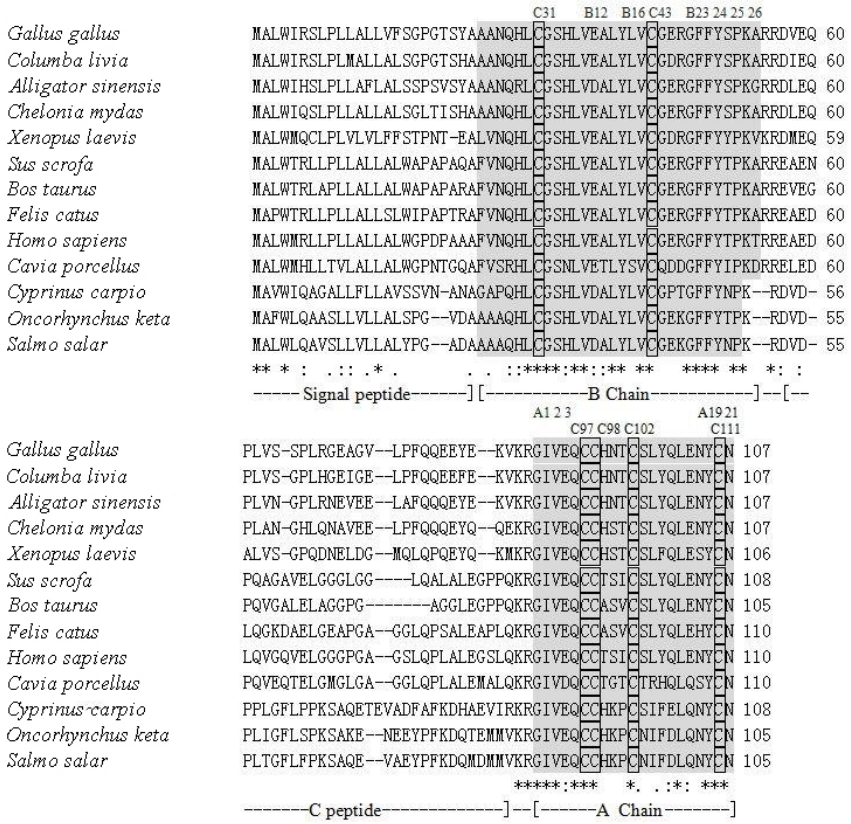

Figure 4. Comparison of preproinsulin amino acid sequences between *Alligator sinensis* and other species.

Preproinsulin structure prediction analysis

According to the Jpred4.1 server, the preproinsulin of *Alligator sinensis* contained 29.90% alpha helix, which included Ile5-Ser18, Ser33-Cys43, Gln91, and Leu99-Asn104. A total of 5.60% beta pleated sheet was found to be located at Ile28-Glu30 and Gln88-Leu90, while 64.48% was aperiodical coil (Figure 5).

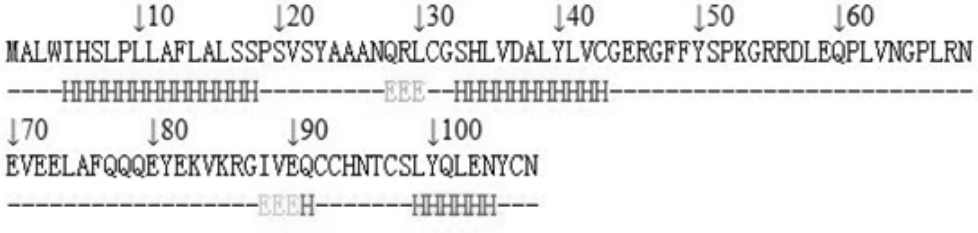

Figure 5. Jpred 4.1 server result for preproinsulin amino acids of *Alligator sinensis*. H = α-helix, E = β-turn, and (-) = random coil.

Phylogenetic analysis

Comparison of the amino acid sequences with other species indicated that the identities of *Alligator sinensis* preproinsulin with reptiles and birds were 81-89%, while with mammals, amphibians, and fishes, values ranged from 48-63% (Table 2). The amino acid sequences of the A-chain were highly conserved, the identities of the A-chain of *Alligator sinensis* preproinsulin with reptiles and birds were 95-100%, and those with mammals, amphibians, and fishes were 62-86% (Table 2). The amino acid sequences in the B-chain were slightly variable, with maximum identities of 87-90% in reptiles and birds. C-peptide had the lowest level of identities among different vertebrates. Sequence comparison of the *Alligator sinensis* and birds, including *Gallus gallus* and *Columba livia*, indicated higher similarity to birds in general, particularly to *Columba livia* (Table 2).

Table 2. Similarity comparison for preproinsulin gene sequences between *Alligator sinensis* and other species.

	Species	GenBank ID	Identities			
			Preproinsulin	B-chain	C-chain	A-chain
Mammals	*Homo sapiens*	NP_001172026.1	67/107 (63%)	24/30 (80%)	-	18/21 (86%)
	Sus scrofa	NP_001103242.1	65/107 (61%)	24/30 (80%)	-	18/21 (86%)
	Felis catus	NP_001009272.1	62/107 (58%)	24/30 (80%)	-	17/21 (81%)
	Bos taurus	NP_776351.2	65/107 (61%)	24/30 (80%)	-	18/21 (86%)
	Cavia porcellus	NP_001166362.1	52/107 (49%)	16/30 (53%)	-	13/21 (62%)
Birds	*Gallus gallus*	NM_205222.2	88/107 (82%)	27/30 (90%)	23/32 (72%)	21/21 (100%)
	Columba livia	EMC88047.1	87/107 (81%)	26/30 (87%)	23/32 (72%)	21/21 (100%)
Reptiles	*Chelonia mydas*	EMP32845.1	96/107 (89%)	27/30 (90%)	24/32 (75%)	20/21 (95%)
Amphibians	*Xenopus laevis*	NP_001079351.1	69/107 (64%)	23/30 (77%)	17/32 (53%)	18/21 (86%)
Fishes	*Oncorhynchus keta*	P04667.2	51/107 (48%)	24/30 (80%)	-	14/21 (67%)
	Salmo salar	ACI69187.1	56/107 (52%)	24/30 (80%)	-	14/21 (67%)
	Cyprinus carpio	P01335.1	60/107 (56%)	21/30 (70%)	-	15/21 (71%)

The phylogenetic tree of proinsulins of *Alligator sinensis* and other vertebrates was based on the alignment amino acid sequences of proinsulin and was derived using the N-J bootstrap method in MEGA 5.0; the numbers at each node represent the bootstrap values. The phylogenetic tree showed that *Alligator sinensis* and *Chelonia mydas* formed a branch, and then clustered with *Gallus gallus* and *Columba livia* (Figure 6). The topology of the tree revealed that the *Alligator sinensis* had a closer relationship with birds.

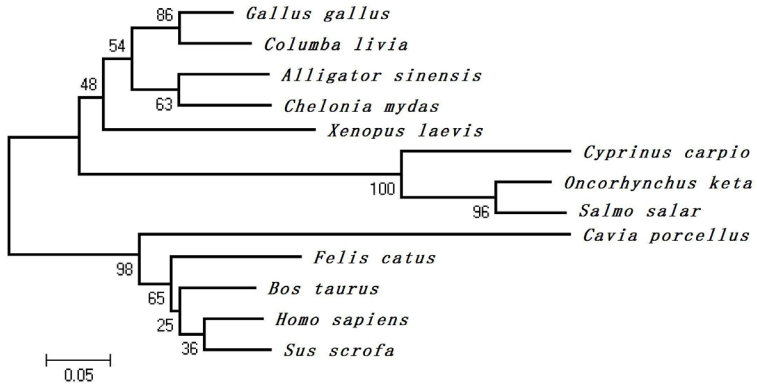

Figure 6. Phylogenetic tree of *Alligator sinensis* and other species proinsulins.

DISCUSSION

In this study, the complete cDNA of *Alligator sinensis* preproinsulin gene was cloned using the RT-PCR and RACE methods. The preproinsulin consists of a signal peptide, mature peptide (A-chain and B-chain), and C-peptide. The amino acid sequence of insulin shared high identity with those of other vertebrates. In a comparison with the preproinsulin amino acid sequence of *Alligator sinensis* with other species, birds and amphibians showed the highest positive rate, while mammals and fishes were the lowest level. Jackintell and Lance (1994) detected insulin-containing cells in the embryonic pancreas of *Alligator mississippiensis*, which first appeared at stage 8. However, Li et al. (2002) did not identify insulin immunoreactive cells in every stage of the embryonic pancreas of *Alligator sinensis* using an immunohistochemical method; they suggested 2 explanations: first, the structure of alligator insulin is sufficiently different from human insulin that it is unrecognizable by anti-human insulin monoclonal antibody. Second, there are no B cells in the pancreas of *Alligator sinensis* and there may be an alternative mechanism for regulating blood glucose level in *Alligator sinensis*. This study verified that insulin was expressed in the pancreas of *Alligator sinensis*; its structure is relatively high variable compared to human insulin, and thus could not be detected using mouse anti-human insulin monoclonal antibody.

Our study demonstrated that the A-chain of preproinsulin was the most conserved, followed by the B-chain and the C-peptide region. Two disulfide bonds were present in the A-chain and B-chain of *Alligator sinensis* and were fully conserved. The length of the insulin A-chain and B-chain were infrequently altered, but in all cases the number and location of the cysteine residues was the same, which plays a key role in maintaining the protein conformation of insulin (Chang et al., 1997). The A-chain of *Alligator sinensis* insulin was consistent with that of birds. However, its amino acid sequence was slightly variable with that of other vertebrates. In the A-chain of *Alligator sinensis*, residues A12-A19 were highly conserved with mammals, while residues A12-A19 showed variability with fishes. We found that the N-terminal A-chain of the *Alligator sinensis* [A1 (G)] was completely consistent with other vertebrates. The N-terminal A-chain of insulin is crucial for maintaining the biological activity of insulin. Removing the glycine residue from the N-terminal A-chain results in a profound loss to biological activity (Blundell et al., 1972). Our results showed that the amino acid sequence of the N-terminal B-chain was less conserved in various species. Three amino acids in the N-terminal B-chain of *Alligator sinensis* insulin were the same as those in birds [B1 (A), B2 (A), and B3 (N)], but these amino acids differed in mammals [B1 (F), B2 (V), and B3 (N)] and fishes[B1 (A), B2 (A), and B3 (A)]. Previous studies have demonstrated that the preproinsulin amino acid sequence of the N-terminal B-chain plays an important role in maintaining the hexamer structures (Conlon et al., 1991). Further investigation is required to determine whether changes in the B-chain affect the formation of hexamer structures. Insulin has more than one binding site for the receptor (De Meyts et al., 1994). The known receptor-binding region at residues B12, B16, B23-26, A1-A5, A19, and A21 were conserved in all sequences (Figure 4). Several sites are involved in maintaining the receptor-binding conformation, including glycine at B23, phenylalanine at B24, isoleucine at A2, valine at A3, and tyrosine at A19. The importance of the 3 insulin disulfides and the aromatic amino acids (B24, B25, and A19) in insulin function are well-established (Murray-Rust et al., 1992). Mutagenesis data indicated that a second receptor-binding surface is present on insulin that involves residues A12

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(S), A13 (L), A17 (E), B10 (H), B13 (D), and B17 (L) (Ward and Lawrence, 2011). Multiple alignment analyses showed that the amino acid sequence of the B-chain of *Alligator sinensis* and birds differed at some sites, such as B13, B21, and B30. Further studies are necessary to determine whether amino acid substitutions in the second receptor-binding site of B13 affect combinations with receptors. C-peptide, a cleavage product from the processing of proinsulin to insulin, showed the lowest level of conservation. This region possesses little, if any, biological activity other than its participation in insulin synthesis. However, injection of human C-peptide prevented or attenuated vascular and neural (electrophysiological) dysfunction and impaired Na^+- and K^+-dependent adenosine triphosphate activity in the tissues of diabetic rats (Ido et al., 1997). Additionally studies should be conducted to determine whether C-peptide has the same biological functions in *Alligator sinensis*.

Hormonal peptides have been examined in several studies to evaluate phylogenetic in other vertebrate species. These studies concluded that low-molecular weight polypeptide hormones are not useful for constructing a phylogenetic tree (Conlon et al., 1990; Agulleiro et al., 1994; Dores et al., 1996). Our study showed that the amino acid sequences of proinsulins, which contain the highly variable C-peptide region, are of greater value in cladistic analysis. The phylogenetic gene tree showed that the proinsulin gene of *Alligator sinensis* is very similar to those in birds, which is consistent with the hypothesis that birds and alligator share a common ancestor.

In summary, our results expand our knowledge of preproinsulin gene phylogenetic evolution. Secondly, the results provide data regarding the relationship between the structure and function of insulin. Finally, our data can be used in further studies on the regulation of metabolism in *Alligator sinensis*.

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