Comparative study of the promotion of porcine fetal fibroblast proliferation by overexpression of two transcriptional variants of *SIRT6*

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Mammalian Sirtuin 6 (*SIRT6*) plays an important role in the transcriptional silencing of silent mating-type loci, telomeres, and rDNA. However, the roles of porcine *SIRT6* in cell proliferation are poorly understood, and a better knowledge of these will help improve our understanding of the biological mechanisms of cell growth and development. In this study, a novel variant of porcine *SIRT6* (*SIRT6* V2) identified by reverse transcription-polymerase chain reaction and BLAST analysis showed a 124-bp deletion compared to wild-type *SIRT6* mRNA (*SIRT6* V1). Two recombinant plasmids overexpressing *SIRT6* V1 and *SIRT6* V2 were produced and their roles in the proliferation of porcine fetal fibroblasts (PFFs) were compared. Cells transfected with *SIRT6* V1 proliferated significantly faster than those transfected with *SIRT6* V2 ($P<0.01$), though both proliferated significantly faster than cells expressing an empty vector ($P<0.01$). These results might be caused by altered proportions of α helices and β sheets in the *SIRT6* V2 structure relative to *SIRT6* V1. These results indicate that overexpression of *SIRT6* V1/V2 was involved in promoting PFF proliferation. Deletion of a 124-bp sequence attenuated the effects of *SIRT6* on cell proliferation, possibly as a result of changes in the proportions of α helices and β sheets in the protein secondary structure.

***SIRT6* variant, overexpression, porcine fetal fibroblast, proliferation, prediction assay**

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Yeast silent information regulator 2 (*SIR2*) possesses NAD^+ -dependent deacetylase and ADP-ribosyltransferase activities, and plays essential roles in transcriptional silencing of silent mating-type loci, telomeres, and rDNA. It also represses recombination, chromosomal instability, and ageing [1–3].

SIRT6 is a distant member of the seven *SIR2*-derived homologs. It is expressed predominantly in nuclei and is highly correlated with heterochromatic regions. As a mono-ADP-ribosyltransferase, *SIRT6* uses NAD^+ as a cosubstrate

to transfer the ^{32}P -label from NAD^+ to m*SIRT6* and histones [4–6]. *SIRT6* harbors low-level deacetylase activity for NAD^+ -dependent deacetylation of non-histone proteins [7] and linking site-specific histones [8–11].

SIRT6 is present in all eukaryotes including mammals [12], and deacetylates histone H3 lysine 9 (H3K9) and histone H3 lysine K56 (H3K56) to maintain dynamic changes in their acetylation levels at the telomeres over cell cycles. Histone deacetylation modifies the telomeric chromatin by preventing chromosomal fusion at the end of telomeres, and negatively regulates ageing-correlated gene-expression programs depending on nuclear factor- κB [8,9,13]. *SIRT6* de-

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iciency in mouse embryonic fibroblasts (MEFs) and embryonic cells (ES) leads to hypersensitivity to DNA damage and genomic instability with increased chromosome fragmentation, detached centromeres, and gaps, indicative of chromosomal aberrations [7]. SIRT6 deficiency also attenuates the double-strand-break repair and the base-excision-repair pathways in MEFs. SIRT6-deleted cells show increased sensitivity to DNA damage and premature senescence syndrome [7]. Inactivation of SIRT6 led to telomere dysfunction and premature senescence in human and mouse cells [9].

Deacetylation of H3K9 and H3K56 by SIRT6 decreases global histone acetylation levels in cells, mainly in S phase and G2, or during early mitosis [8,9,13]. Mammalian SIRT6-deficient MEFs and ES cells grew more slowly than wild-type cells in culture, and deletion of SIRT6 led to a reduced proliferation rate in several cell types [7,14].

However, the mechanisms that mediate the effects of porcine SIRT6 on cell proliferation remain poorly understood. A comparison between the effects of different variants on cell proliferation will help to clarify the role of SIRT6 in the physiological regulation of cell growth and development. In this study, we identified a novel porcine SIRT6 variant and constructed plasmids to express the wild-type and novel SIRT6 variants, thus allowing the effects of both variants on porcine fetal fibroblast (PFF) proliferation to be compared.

1 Materials and methods

1.1 Reverse transcription-polymerase chain reaction (PCR)

All animal procedures were approved by the Shanghai Care and Use Committee, China. Heart, liver, spleen, lung, kidney, stomach, lymph nodes, *longissimus dorsi*, fat, large intestine, and cerebral tissues were harvested from Large White pigs. Total RNA was isolated from each tissue using Trizol reagent (Invitrogen, USA). cDNA was synthesized for reverse transcription-PCR from less than 5 µg of each total RNA, according to the manufacturer's protocol (TaKaRa, Japan). cDNA samples (5 µL) of the same concentration were pooled together to use as a template.

Porcine SIRT6 cDNA containing a complete or partial open reading frame (ORF) was cloned by reverse transcription-PCR using the primer S1 (Table 1). cDNAs containing complete or partial ORFs were named SIRT6 variant 1 (SIRT6 V1, wild-type SIRT6 mRNA) and SIRT6 variant 2 (SIRT6 V2), respectively. The reaction was carried out in a volume of 25 µL for 5 min at 94°C, 32 cycles of 30 s at 94°C, 30 s at 60°C, and 40 s at 72°C, ending with 8 min at 72°C, with 16°C hold. All PCR products were purified using a MinElute Gel Extraction Kit (Qiagen, USA) and cloned into the pMD18-T vector (TaKaRa) for sequencing.

1.2 Recombinant plasmid production for overexpression of SIRT6 variants

Two cDNA fragments of the porcine SIRT6 variants were amplified using the purified cDNA fragments as a template. The primers used were: forward primer 5'-TTTGAA-TTCGCTGGGAATCACCTCCGCCTT-3' (*Xho* I, underlined), and reverse primer 5'-TATCTCGAGGTGAGGATGTCGGTGAATTATGCG-3' (*Eco*R I, underlined). The fragments were cloned into the pMD18-T vector and released by restriction with *Xho* I and *Eco*R I (TaKaRa). The purified fragments were inserted into the vector pcDNA3.1(-) (Invitrogen) using T4 DNA ligase (TaKaRa) at the *Xho* I/*Eco*R I site. The constructed plasmids were named pcDNA3.1(-)-SIRT6 V1 and V2, and were identified by restriction profiling and DNA sequencing.

1.3 Isolation, culture and transfection of PFFs

The isolation of PFFs was performed as described previously [15]. Thawed cells were seeded into 25 cm² flasks for expansion. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with penicillin (100 IU/mL; Sigma, USA), streptomycin (100 mg/mL; Sigma), and 10% fetal bovine serum (Invitrogen), and maintained at 37°C with 5% CO₂ humidified air. PFFs were passaged at 95% confluence. The plasmids [pcDNA3.1 (-)-SIRT6 V1/V2 and empty vector] were transfected using Lipofectamine 2000 (Sigma).

1.4 Quantitative real-time PCR (qRT-PCR)

Total RNA extraction and cDNA synthesis were carried out as described previously [16]. qRT-PCR was performed using SYBR Green (TaKaRa) and the ABI Prism 7000 sequence detection system (Applied Biosystems, USA). The primers S2 (Table 1) used to amplify SIRT6 V1/V2 were synthesized according to the shared coding region of both variants, with an amplicon of 248 bp. The expression of the porcine β -actin gene was evaluated as an internal control. The β -actin primer was 146 bp long (Table 1).

Table 1 The primers for amplification of SIRT6 variants cDNA and qRT-PCR

Name	Primer sequences (5'→3')	T _m ^{a)} (°C)	PCR product length (bp)
S1	GGCGGTGAGGATGTCGGTGAA GGAGGGACTGTGAGGCAATT	60	1490/1366
S2	AAGGGCAAGTGCGGTCT GCCATGTGGGTCTTCTGTG	58.3	248
β -actin	TGCGGCATCCACGAACTAC AGGGCCGTGATCTCTTCTG	58.3	146

a) T_m, optimal annealing temperature.

The amplification reaction was initiated by pre-incubation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58.3°C for 30 s, and extension at 72°C for 40 s, performed in a 20 μ L volume containing 10 μ L SYBR Premix EX Taq GC (2 $\times$), 0.4 μ L of each PCR primer, 0.4 μ L ROX reference dye (50 $\times$), and 0.03 μ g cDNA. The relative expression levels were calculated using the $2^{-\Delta\Delta C_T}$ method, as described previously [17].

1.5 Western blot analysis

Antibodies specific for SIRT6 were raised against the following peptide sequence: TMEERGLAPKFDITTFESARPT-QTHMALVQLERVGLLRFLVSQNVDGLHVR. This sequence represents a region of human SIRT6 with 96% similarity with the corresponding region of porcine SIRT6. Western blot analysis was performed as described previously [18]. Briefly, western blots were incubated overnight at 4°C with primary antibody (1:700; Sigma) to porcine SIRT6, anti-glyceraldehyde phosphate 3-dehydrogenase (GAPDH) antibody (1:10000; Sigma) in phosphate-buffered saline (PBS) containing 0.05% Tween 20 with 5% bovine serum albumin. Blots were then incubated with goat anti-rabbit horseradish peroxidase-conjugated second antibody (1:5000; Sigma) in 5% non-fat milk for 1 h at room temperature. The primary antibodies were detected by anti-rabbit horseradish peroxidase-conjugated second antibody, using anti-GAPDH as an internal control.

1.6 Cell counts and determination of population doubling times (PDs)

The harvested cells were seeded into 75 cm² flasks at 20000 cells per flask and cultured for 3, 6, 9, 12, and 15 d, respectively. All experiments were repeated three times. The medium containing G418 was changed every 3 d. The cells were removed at the end of each culture time and dissolved in 2 mL medium containing DMEM and fetal calf serum. The cells were counted using a hemocytometer. The average values were calculated using the formula: $AVE = (n_1 + n_2 + n_3)/3$, where n_1 , n_2 , and n_3 were the counted values.

PDs were calculated according to the formula: $PD = (\log Nd - \log Ni) / \log 2$, where Nd is the average number of cells counted, and Ni is the number of cells seeded. A

growth curve was constructed according to the numbers of PDs [19]. PD analysis was used to assess the effects of both *SIRT6* variants on PFFs in culture. PDs of control cells were also calculated and used as a loading control.

1.7 Statistical analysis

The significance of differences in cell proliferation and qRT-PCR results among cells transfected with empty vector, *SIRT6* V1 and *SIRT6* V2 in five separate experiments were identified by one-way analysis of variance with a Benjamini and Hochberg false discovery rate (BH-FDR = 0.05) for multiple testing correction, followed by Tukey's *post-hoc* tests using SPSS 10.0 software. The difference was considered significant at $P < 0.05$, and extremely significant at $P < 0.01$. All the data were presented as mean $\pm$ SEM.

1.8 Prediction of SIRT6 variant protein structures

The deleted sequences in the *SIRT6* V2 mRNA relative to *SIRT6* V1 mRNA were analyzed using a multiple sequence alignment program (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The coding sequences and corresponding amino acid sequences of both *SIRT6* variants were predicted using a graphical analysis tool (<http://www.ncbi.nlm.nih.gov/guide/all/>) and the homology domains of both variant proteins were aligned using ClustalW. Version 2.0 of the Phyre server (<http://www.sbg.bio.ic.ac.uk/servers/phyre/>) was used to predict the structures of both SIRT6 proteins [20,21].

2 Results and discussion

2.1 Identification and analysis of a novel variant of porcine SIRT6

Two porcine *SIRT6* variants have previously been isolated, one with a 19-bp deletion in exon 1 and a 327-bp deletion in exon 8, and the other with a 36-bp deletion in exon 1 and a 291-bp deletion in exon 8 [22]. In this study, reverse transcription-PCR analysis of *SIRT6* transcripts yielded another novel *SIRT6* variant (Figure 1, not submitted to GenBank) compared to wild-type *SIRT6* (GenBank accession: FJ457624) and expressed sequence tags in GenBank. This new variant was determined to have a deletion of 124 bp using BLAST, relative to the gene sequence of porcine

Figure 1 Identification and analysis of a novel variant of porcine *SIRT6*. Both *SIRT6* variant mRNAs are shown relative to the gene sequence of porcine *SIRT6*. The exons are shaded black, except for exon 7 (124 bp), which is shaded white and was deleted in *SIRT6* V2 (data not shown, GenBank accession number of the cDNA sequence was not obtained). *SIRT6*, Sirtuin 6; *SIRT6* V1, wild-type *SIRT6* mRNA; *SIRT6* V2, novel variant of porcine *SIRT6*.

SIRT6 (GenBank accession: HQ242712, submitted by our laboratory), which represents the complete exon 7 in *SIRT6* V1.

2.2 Construction of PFFs overexpressing *SIRT6* V1 and *SIRT6* V2

cDNA sequences for *SIRT6* V1 and V2 were amplified using primer S1, and cloned into the PMD18-T vector for sequencing. Fragments of 1490 and 1366 bp were subcloned into pcDNA3.1 (–) to produce pcDNA3.1(–)-*SIRT6* V1 and pcDNA3.1(–)-*SIRT6* V2. Both recombinant plasmids were identified with *Xho* I/*Eco* R I (Figure 2) and by DNA sequencing (data not shown).

In order to investigate the differences in cell proliferation rates between the cells transfected with *SIRT6* V1 and *SIRT6* V2, we generated PFF lines stably overexpressing each variant. No visible cell death or growth retardation was observed, and the cells became G418-resistant after 2 weeks using G418 at 500 $\mu\text{g mL}^{-1}$. qRT-PCR showed upregulation of both *SIRT6* variant mRNAs in these cells, to levels at least 5-fold higher than the endogenous expression in cells transfected with empty control vector. Six overexpressing clones were selected for each of *SIRT6* V1 and V2 and designated RM1–RM6 and RN1–RN6, respectively (Figure 3(a)). Clones RM1 and RN4 showed similar mRNA abundances and were used for further studies. Overexpression of both *SIRT6* variants was also confirmed by Western blot analysis (Figure 3(b)). The same antibody raised against the shared region of *SIRT6* V1 and *SIRT6* V2 was used in the two independent experiments. The results showed more intense *SIRT6* V1 and V2 protein bands in the RM1 and RN4 clones than in the control cells. *SIRT6* proteins encoded by *SIRT6* V1 and *SIRT6* V2 hybridized to produce two bands, as seen in previous experiments [5,11,23].

Figure 2 Identification of plasmids of both *SIRT6* variants by restriction analysis. Lanes 1, 2: the recombinant plasmids pcDNA3.1(–)-*SIRT6* V2 and pcDNA3.1(–)-*SIRT6* V1 were restricted with *Xho* I/*Eco* R I. Fragments of 5500, 956, and 1080 bp were produced. Lane 3: empty-vector pcDNA3.1 (–) was used as a loading control. Lane M: 5000 bp marker.

Figure 3 Construction of PFFs (porcine fetal fibroblasts) overexpressing *SIRT6* V1 and V2. (a) *SIRT6* mRNA expression was examined in six experimental clones transfected with *SIRT6* V1 (RM1–RM6) and six transfected with *SIRT6* V2 (RN1–RN6) by qRT-PCR. PFFs transfected with empty vector were examined for endogenous expression and served as a loading control. (b) Western blot assay was carried out to identify overexpression in RM1 cells transfected with *SIRT6* V1 and RN4 cells transfected with *SIRT6* V2. Human *SIRT6* antibody was raised against the shared region of both *SIRT6* variants proteins. GAPDH was used as a loading control.

2.3 Comparison of proliferation promotion by *SIRT6* V1 and *SIRT6* V2

Data were collected at different culture time points. Average cell counts at each time point were derived using the AVE formula for the cells expressing both variants and the empty vector, and PDs were calculated. A growth curve was constructed based on the various PDs (Figure 4). The PDs increased with culture time for all cell lines, and the increase was very slow for the initial 3 d. From the third day to the end of the culture period, the rates of increase of RM1 and RN4 cells were higher than that of the control cells, with RM1 cells having the highest increase rate. Control, RM1, and RN4 cells increased by 6.65, 8.61, and 7.51 PDs, respectively. These results indicate that the cells expressing *SIRT6* V1 and *SIRT6* V2 proliferated faster than the control cells, by 1.96 and 0.86 PDs, respectively ($P < 0.01$). Cells transfected with *SIRT6* V1 proliferated 1.1 PDs faster than cells with *SIRT6* V2 over the 15 d culture period ($P < 0.01$).

2.4 Prediction of *SIRT6* V1 and V2 protein structures

The coding sequences of *SIRT6* V1 and *SIRT6* V2 mRNAs with the initiation methionine were predicted to span 1080 and 651 bp, respectively. The corresponding amino acid sequences contained 359 and 216 amino acids (data not

Figure 4 PDs (population doublings) determination and comparison of proliferation promotion between *SIRT6* variants. PDs were determined at different culture time points, and growth curves were constructed based on number of days in culture. Cells transfected with either *SIRT6* variant proliferated significantly faster than control cells ($P < 0.01$). The proliferation rate of the cells with *SIRT6* V1 was significantly higher than that of the cells with *SIRT6* V2 ($P < 0.01$). Growth curves were constructed for control, *SIRT6* V1, and *SIRT6* V2 cells, respectively.

shown). The predicted structure of *SIRT6* V1 was mainly composed of 108 α helices (31.5%) and 89 β sheets (25.9%), as shown in Figure 5(a), while the predicted structure of *SIRT6* V2 was mainly composed of 78 α helices (39.0%) and 59 β sheets (29.5%), as shown in Figure 5(b).

α Helices and β sheets are the two most common types of secondary structure, and combine in different ways to create different spatial arrangements of polypeptide chains [24,25], thus playing essential roles in guiding and promoting the early steps described in many folding models [26,27]. Stabilizing mutations of the helix accelerate folding, while destabilizing mutations slow folding [28]. Destabilizing of the helix in protein L is the rate-limiting step in folding and unfolding, with a more significant influence on the unfolding than on the folding rate. Hydrophobic interactions and

the residues in the helix are important factors in the rate-limiting step in folding [29]. A study of the mechanisms of β sheets identified the special spatial formation as the rate-limiting step in β -sheet folding [30]. The proportions of α helices and β sheets in the predicted structure of *SIRT6* V2 were higher than those in the *SIRT6* V1 protein (Figure 5), as a result of deletion of the 124-bp sequence. This might change the interactions of the secondary structures and influence their folding and unfolding rates, which in turn determine the specific tertiary structure [24,25]. In addition, all aspects of cell metabolism are catalyzed by enzymes, many of which depend on cofactors. The active configuration is produced via the combination of enzyme and cofactor, which usually includes an active site that the substrate can interact with [31]. Many enzymes are selective for just one substrate, and their activity is inhibited if the active site is blocked or the shape of the substrate is changed [32,33]. The change in structure of *SIRT6* V2 due to the deleted 124-bp sequence (Figure 5) might thus alter the interactions between substrate and enzyme, which are involved in the series of reactions associated with cell proliferation. The altered enzyme may provide an active site that binds inhibitors more easily than substrates, or may reduce the active site thus attenuating the catalytic efficiency of the enzyme [34]. These changes might decrease the efficiency of *SIRT6* variants in terms of the promotion of PFF proliferation.

In summary, overexpression of both *SIRT6* variants demonstrated their involvement in the regulation of PFF proliferation. The promotion efficiency of *SIRT6* V1 overexpression was significantly higher than that of *SIRT6* V2 ($P < 0.01$). Deletion of a 124-bp sequence (exon 7) in the ORF of *SIRT6* attenuated its ability to promote PFF proliferation. It is possible that the altered proportions of α helices and β sheets in the *SIRT6* V2 protein structure, relative

Figure 5 Comparison of predicted *SIRT6* V1 and *SIRT6* V2 protein structures. The protein structures of *SIRT6* V1 and V2 were predicted and compared. (a) Predicted structure of *SIRT6* V1 was composed mainly of 108 α helices and 89 β sheets, which comprised 31.5% and 25.9% of the polypeptide, respectively. (b) Predicted structure of *SIRT6* V2 was composed mainly of 78 α helices and 59 β sheets, comprising 39.0% and 29.5% of the polypeptide, respectively.

to SIRT6 V1, may have changed their interactions and altered the folding and unfolding rates, or attenuated the catalytic efficiency of the enzymes associated with cell proliferation.

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- 1 Landry J, Sutton A, Tafrov S T, et al. The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proc Natl Acad Sci USA*, 2000, 97: 5807–5811
- 2 Kaeberlein M, McVey M, Guarente L. The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes Dev*, 1999, 13: 2570–2580
- 3 Moazed D. Enzymatic activities of Sir2 and chromatin silencing. *Curr Opin Cell Biol*, 2001, 13: 232–238
- 4 Liszt G, Ford E, Kurtev M, et al. Mouse Sir2 homolog SIRT6 is a nuclear ADP-ribosyltransferase. *J Biol Chem*, 2005, 280: 21313–21320
- 5 Michishita E, Park J Y, Burnes J M, et al. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Mol Biol Cell*, 2005, 16: 4623–4635
- 6 Du J, Jiang H, Lin H. Investigating the ADP-ribosyltransferase activity of sirtuins with nad analogues and 32P-NAD. *Biochemistry*, 2009, 48: 2878–2890
- 7 Mostoslavsky R, Chua K F, Lombard D B, et al. Genomic instability and ageing-like phenotype in the absence of mammalian SIRT6. *Cell*, 2006, 124: 315–329
- 8 Kawahara T L, Michishita E, Adler A S, et al. SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. *Cell*, 2009, 136: 62–74
- 9 Michishita E, McCord R A, Berber E, et al. SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. *Nature*, 2008, 452: 492–496
- 10 Zhong L, D'Urso A, Toiber D, et al. The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. *Cell*, 2010, 140: 280–293
- 11 Tennen R I, Berber E, Chua K F. Functional dissection of SIRT6: Identification of domains that regulate histone deacetylase activity and chromatin localization. *Mech Ageing Dev*, 2010, 131: 185–192
- 12 Frye R A. Characterization of five human cDNAs with homology to the yeast *SIR2* gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. *Biochem Biophys Res Commun*, 1999, 260: 273–279
- 13 Michishita E, McCord R A, Boxer L D, et al. Cell cycle-dependent deacetylation of telomeric histone H3 lysine K56 by human SIRT6. *Cell Cycle*, 2009, 8: 2664–2666
- 14 Lombard D B, Schwer B, Alt F W, et al. SIRT6 in DNA repair, metabolism and ageing. *J Intern Med*, 2008, 263: 128–141
- 15 Boquest A C, Day B N, Prather R S. Flow cytometric cell cycle analysis of cultured porcine fetal fibroblast cells. *Biol Reprod*, 1999, 60: 1013–1019
- 16 Wang M H, Long M, Zhu B Y, et al. Effects of sargentgloryvine stem extracts on HepG-2 cells *in vitro* and *in vivo*. *World J Gastroentero*, 2011, 17: 2848
- 17 Schmittgen T D, Livak K J. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc*, 2008, 3: 1101–1108
- 18 Trog D, Moenkemann H, Haertel N, et al. Expression of ABC-1 transporter is elevated in human glioma cells under irradiation and temozolomide treatment. *Amino Acids*, 2005, 28: 213–219
- 19 Blander G, Bhimavarapu A, Mammone T, et al. SIRT1 promotes differentiation of normal human keratinocytes. *J Mol Biol*, 2008, 129: 41–49
- 20 Kelley L A, Sternberg M. Protein structure prediction on the Web: A case study using the Phyre server. *Nat Protoc*, 2009, 4: 363–371
- 21 Garnier J, Osguthorpe D, Robson B. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J Mol Biol*, 1978, 120: 97–120
- 22 Jin D, Tan H J, Lei T, et al. Molecular cloning and characterization of porcine sirtuin genes. *Comp Biochem Physiol B Biochem Mol Biol*, 2009, 153: 348–358
- 23 Kanfi Y, Shalman R, Peshti V, et al. Regulation of SIRT6 protein levels by nutrient availability. *FEBS Lett*, 2008, 582: 543–548
- 24 Branden C, Tooze J. Introduction to Protein Structure. New York: Garland Pub. Inc., 1998
- 25 Berman H M, Goodsell D S, Bourne P. Protein structures: From famine to feast. *Am Sci*, 2002, 90: 350
- 26 Kim P S, Baldwin R L. Intermediates in the folding reactions of small proteins. *Annu Rev Biochem*, 1990, 59: 631–660
- 27 Karplus M, Weaver D L. Protein folding dynamics: The diffusion-collision model and experimental data. *Protein Sci*, 1994, 3: 650–668
- 28 Sosnick T R, Jackson S, Wilk R R, et al. The role of helix formation in the folding of a fully alpha-helical coiled coil. *Proteins*, 1996, 24: 427–432
- 29 Kim D E, Yi Q, Gladwin S T, et al. The single helix in protein L is largely disrupted at the rate-limiting step in folding. *J Mol Biol*, 1998, 284: 807–815
- 30 Deechongkit S, Nguyen H, Jager M, et al. Beta-Sheet folding mechanisms from perturbation energetics. *Curr Opin Struc Biol*, 2006, 16: 94–101
- 31 Zimmerman J. Enzyme kinetics and mechanism. *Biochem Mol Biol Edu*, 2007, 35: 386–388
- 32 Rabinovich M, Melnick M, Bolobova A. The structure and mechanism of action of cellulolytic enzymes. *Biochem (Moscow)*, 2002, 67: 850–871
- 33 Page M, Di Cera E. Serine peptidases: Classification, structure and function. *Cell Mol Life Sci*, 2008, 65: 1220–1236
- 34 Price N C. What is meant by 'competitive inhibition'? *Trends Biochem Sci*, 1979, 4: N272–N273

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