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重组蛋白质生产技术

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摘要: 在后基因组时代,对于抗体生产、生化活性研究和蛋白质结构测定等研究领域而言,重组蛋白质生产是一类重要的技术。重组蛋白质生产过程中的一些限制因素包括蛋白质表达水平低,蛋白质沉淀和在纯化过程中蛋白质生化活性损失等。讨论提高重组蛋白质生产的几种方法,包括:(1)选择高水平蛋白质表达系统;(2)优化蛋白质表达条件;(3)尽量减少蛋白质纯化步骤;(4)维持蛋白质结构和功能。最后举例讨论抗炎蛋白质 Tristetraprolin 在大肠杆菌和人类细胞中的表达和纯化。

关键词: 重组蛋白质; 蛋白质表达; 蛋白质纯化; 大肠杆菌; 酵母; 人类细胞
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Recombinant Protein Production Technology

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Abstract: Recombinant protein production is an important technology for antibody production, biochemical activity study, and structural determination of proteins during the post - genomic era. Limiting factors in recombinant protein production include low - level protein expression, protein precipitation, and loss of protein activity during the purification processes. This paper discusses several approaches to improve recombinant protein production, including 1) selecting high - level protein expression systems; 2) optimizing protein expression conditions; 3) minimizing protein purification steps; 4) maintaining protein structure and function. Finally, the review presents an example for the expression and purification of the anti - inflammatory protein tristetraprolin from *E. coli* and human cells.

Key words: recombinant protein; protein expression; protein purification; *E. coli*; yeast; human cells

1 Introduction

The DNA sequences of many organisms including human, mouse, *Arabidopsis*, and rice have been known

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在植物科学、生物化学、分子生物学、营养学和生物技术等领域有广泛研究,对生物蛋白酶的表达、纯化、结构、功能、酶学、蛋白组学、生化遗传及代谢途径等领域有深入研究。先后在 25 种科技期刊、8 种图书和诸多国际会议论文集中发表论文 100 余篇。被 SCI 刊物收录的硕士论文同时被 10 多种最著名植物科学期刊和多种图书引用。1994 年被美国体外生物学协会授予 Earle 奖。E-mail: Heping.Cao@ars.usda.gov; peacetd2003@yahoo.com。

for many years. However, the functions of a large percentage of the genes are not clear. The immediate challenge of post-genomic biology is to determine the biological functions of proteins coded for by the genomes. Many endogenous proteins occur in extremely low-abundance and are labile, which are the major problems in the determination of the functions of those proteins.

Recombinant proteins can be used as an alternative source for endogenous proteins. Production of active proteins in large quantities is necessary for the study of protein structure and function. Purified recombinant proteins are also important for the production of antibodies and pharmaceutical reagents. However, a great number of proteins are difficult to express and purify from any source. These proteins include membrane proteins, lipid-associated proteins, and low-abundance proteins. The causes of the difficulties in protein expression and purification are various, such as protein insolubility, protein degradation, and low-level of protein expression. Therefore, production of high-quality recombinant protein requires consistent optimization of protein expression and purification procedures.

Many approaches have been used in our studies to address the detailed relationship between the structure and the function of proteins involved in metabolic pathways and signal transduction pathways (Table 1)[1-10]. Typically, the putative functions of the proteins of interest are initially proposed based on comprehensive analyses of similar sequences deposited in the GenBank databases[1,11]. Then, the proteins of interest are expressed in cells including *E. coli*[1,2,11] and human cells[3,10] and purified from the over-expressed cells free of contaminated

表 1　重组蛋白质在不同系统中的表达和纯化

Tab. 1　A number of recombinant proteins were expressed and purified using various expression systems

Protein	Origin	Host	Fusion/tag	Purification	Purpose	Reference
Anti-inflammatory and mRNA-binding protein Tristetraprolin (TTP/ZFP36)	Human Mouse	*E. coli* Human cells	MBP NusA GST His-tag	Affinity Convention	Antibody production Immunoblotting Immunocytochemistry Immunoprecipitation RNA-binding activity Phosphorylation sites	[3,14] [3,10,14,36-38] [3,8,10] [2,3,6,7,14] [2,3,5,6,10,35]
TTP homologue (TIS11B/ZFP36L1)	Mouse	*E. coli*	MBP	Affinity Convention	Antibody production Immunoblotting	[7] [7]
Adenylate translocator (BT1)	Maize (Corn)	*E. coli*	GST	Affinity	Antibody production Immunoblotting	[45,46] [45-49]
Starch synthases (SS)	Maize (Corn)	*E. coli* Yeast	GST S-tag T_7-tag Flag	Affinity Convention	Antibody production Immunoblotting Immunoprecipitation Activity	[1] [1,21] [1,21] [1,21]
Starch branching enzymes(SBE)	Maize (Corn)	*E. coli*	Native S-tag T_7-tag	Affinity Convention	Activity Chemical modification	[12,13] [12,13]
Starch debranching enzymes (SDBE)	Maize (Corn)	*E. coli*	GST	Affinity Convention	Antibody production Immunoblotting	[11] [11]
Sterol methyltransferase (SMT)	Yeast	*E. coli*	Native	Convention	Activity	unpublished
Diacylglycerol acyltransferase (DGAT)	Tung tree	*E. coli* Yeast	MBP HA	Affinity Convention	Antibody production Activity	unpublished [9]

factors [2-3,10,12-13]. Finally, the purified proteins are used for generating antibodies [1,3,7,11,14] and biochemical characterization including enzymatic activities, post-translational modifications, and structure determination [1,3,5,8,10,11,14].

We have expressed and purified a large number of proteins and their mutant derivatives in *E. coli*, yeast, and human embryonic kidney 293 cells (Table 1). Figure 1 shows an example of recombinant protein expression and purification in both *E. coli* and human cells for facilitating the understanding of various issues discussed in the paper [2-3]. Some of the approaches described below have been used in our studies to increase recombinant protein production. Although most of the discussion is focused on recombinant protein production in *E. coli*, the approaches discussed in the paper should be applicable to other expression systems.

2 Approaches to improve recombinan protein production

2.1 Select high level protein expression systems

Many recombinant protein expression systems have been developed over the years. The widely used expression hosts are *E. coli*, yeast, insect and mammalian cells. New methods have been developed recently using farm animals and crops for large scale production of recombinant proteins and vaccines. Selection of expression systems depends on the objectives of experiments. For proteins without post-translational modifications or proteins to be purified as antigens, the *E. coli* expression system is the method of choice. For functional studies, if the proteins of interests are known to be modified in vivo, eukaryotic systems should be used for expression of the proteins.

We have expressed plant, animal, yeast, and human proteins using *E. coli*, yeast, and human cells as hosts (Table 1). The expression systems we examined include proteins of interests fused to *E. coli* maltose binding protein (MBP), *Schistosoma japonicum* glutathione-S-transferase (GST), NusA, His-tag, S-tag, T_7-tag, HA-tag, and Flag-tag, as well as no-tag at all. While many proteins could be easily expressed with His-tag and GST fusion protein systems, some proteins are particularly difficult to express in these expression systems. One example is the anti-inflammatory mRNA binding and destabilization protein tristetraprolin/zinc-finger protein 36 (TTP/ZFP36) [4,15-18]. Previous studies showed that recombinant TTP fused to GST was extensively degraded and mostly precipitated [2,19-20]. We showed that MBP and NusA expression systems produced more soluble TTP fusion protein as compared to the other systems tested [2-3,14]. Another example is the maize starch synthase III Dull1 (DU1) protein [21-22]. The amino-terminal region of DU1 protein was abundantly expressed and easily purified to homogeneity with GST fusion system, but the level of expression with S-tag or T_7-tag in the pET system was minimal [1]. On the contrary, pET system expressed large quantities of maize starch branching enzymes in *E. coli* [12-13,23]. These studies suggest that the expression levels of recombinant proteins depend on the proteins to be expressed. Therefore, it is important to test several expression systems for new protein expression.

2.2 Optimize protein expression conditions

Many over-expressed proteins are largely insoluble, a major problem decreasing protein purification yield. Several approaches have been used to reduce this problem, such as selection of fusion partners that have been shown to enhance the solubility of the proteins of interest, reduction of the temperature for protein induction, variation of isopropyl β-D-1-thiogalactopyranoside (IPTG) induction time, and optimization of culture medium.

One of the widely used methods to increase protein solubility is to select fusion partners that can enhance the solubility of the proteins of interest. For example, TTP expressed in *E. coli* as a GST fusion protein was shown to be extensively degraded and mostly insoluble [19-20]. It has been reported that *E. coli* MBP and NusA promote the solubility of polypeptides to which they are fused [24-25]. We therefore investigated the possibility of expressing recombinant TTP using MBP and NusA fusion systems. Immunoblotting analysis showed that MBP-TTP and NusA-TTP were degraded much less than GST-TTP in the over-expressed *E. coli* [2,4].

The temperature used to induce recombinant protein expression also contributes to protein solubility. Recombinant proteins are typically expressed in *E. coli* at 37 ℃. However, lowering induction temperature is an effective method for producing soluble proteins [2,7]. Under lower temperature, recombinant proteins are induced

slower in cells with less precipitation. We found that IPTG induction at 30 ℃ was optimal for the expression of soluble GST – TTP [2] and GST – ZPU1, a pullulanase – type starch debranching enzyme from maize [11]. Similarly, MBP – ZFP36L1 protein, a TTP homologue, was expressed much more under 25 ℃ and 30 ℃ than that under 37 ℃ [7]. Starch branching enzymes were induced overnight at 25 ℃ routinely for active protein production [12-13].

IPTG induction time is another factor which needs to be evaluated in any scheme for high level expression of soluble proteins. TTP is optimally expressed by IPTG induction for 2 – 4 hours [2]. However, starch branching enzyme induction was found to be optimal overnight at 25 ℃ [12,13,26-27].

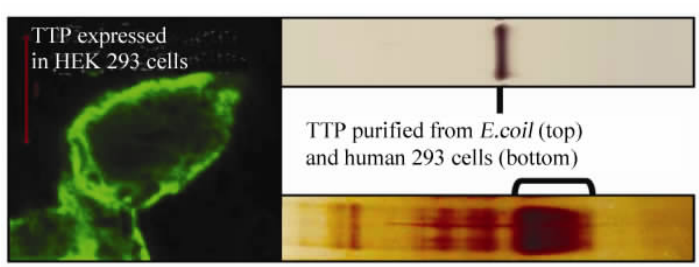
Optimization of culture medium is another way to improve soluble protein expression. A number of studies have shown that sorbitol in culture medium results in more soluble proteins recovered from over-expressed cells, probably due to its ability to slow down cell growth. For example, we used a medium containing 1 mol/L sorbitol to express GST – ZPU1 protein, which was subsequently purified and used as antigens for antibody production [11].

2.3 Minimize protein purification steps

Protein purification yield is reduced significantly after each purification step. Therefore, purification scheme should be designed to use as few steps as possible. Since recombinant proteins are usually expressed as fusion proteins, affinity purification should be used as the first step of purification. Many recombinant proteins could be purified to near homogeneity by this step alone. GST – DU1 amino terminal domain was affinity – purified to near homogeneity and used for antibody production [1]. For difficult – to – purify proteins, however, additional steps are required to purify the proteins to satisfactory. TTP is extremely precipitated in over – expressed *E. coli*. MBP – TTP could be purified to 70% homogeneity by amylose resin affinity step [2]. Additional steps such as Superose 12 size exclusion and MonoQ anion exchange chromatography are required to purify MBP – TTP to near homogeneity for antibody production and functional studies [2-3,14].

When fusion tag is not used, the expressed protein is purified by a series of chromatographic steps. For example, starch branching enzymes were expressed as native proteins and purified by ammonium sulfate precipitation, DEAE – Sepharose fast flow, hydrophobic interaction, and MonoQ anion exchange columns [12-13].

When fusion tag is used and the purpose of the expressed protein is for functional studies, the fusion partners may need to be removed beforehand. This is normally done by digestion of the fusion proteins with appropriate proteases followed by additional purification steps to remove the fusion partners and the proteases. Digestion conditions such as time and temperature used for digestion need to be determined by detailed analyses for each protease and each recombinant fusion protein. For example, a PreScission protease recognition site was inserted between MBP and TTP to facilitate purification of TTP from the fusion protein [2]. MBP – TTP was bound to amylose resin and eluted out with maltose. MBP – TTP eluted from amylose resin affinity column was digested with PreScission



(Left) His – TTP was expressed in human embryonic kidney 293 cells. The cells were transfected with plasmid pHis – TTP. His – TTP was transiently expressed in the cells and detected by confocal microscopy using TTP antibodies raised against recombinant MBP – TTP fusion protein purified from over – expressed *E. coli* [3]. (Top right) TTP was purified from over – expressed *E. coli* following protease digestion of MBP – TTP fusion protein. (Bottom right) His – TTP was purified from transfected human embryonic kidney 293 cells. The purified proteins were separated by SDS – PAGE and detected by silver staining (Modified from [3]).

图 1　重组蛋白质 TTP 在大肠杆菌和人类细胞中的表达和纯化

Fig. 1　Expression and purification of recombinant fusion protein TTP using *E. coli* and human cells

protease [2]. However, the same protease performed poorly for the digestion of GST – TTP, probably due to more extensive precipitation of GST – TTP than MBP – TTP [2]. Extensive efforts were directed to purify the released TTP from the digestion mixture by conventional chromatographic procedures, including MonoQ anion exchange, Superdex 75, and Superose 12 size exclusion columns. We were unable to purify TTP in this way due to its extensive precipitation following protease digestion [4]. Since TTP was clearly separated from MBP on SDS – PAGE gel, we purified TTP from the PreScission protease – digested mixture using continuous – elution gel electrophoresis technique [2,4] (Figure 1).

2.4 Maintain protein structures and functions

Structural integrity of the purified proteins is essential for structural and functional studies. This could be assessed by determining the amino and carboxyl-terminal sequences and peptide mapping. The results from these analyses serve as important quality controls. For example, the purified TTP was determined by MALDI – TOF mass spectrometry and N-terminal sequencing [2-3]. The first 13 amino acid residues of TTP were determined to be GPDLTAIYESLLS [2]. The first two amino acid residues (GP) were derived from the PreScission protease site in the vector following PreScission protease digestion. The third sequenced residue (D) was the second amino acid residue of the deduced human TTP amino acid sequence. These sequenced residues were in agreement with deduced amino acid residues and with the PreScission protease digestion site. Protein sequencing and mass spectrometry analyses also identified the integrity of the carboxyl terminal of His – TTP purified from transfected human cells [3].

The functions of recombinant proteins are analyzed using proteins from each purification step, especially the final purified proteins. It is relatively easy to monitor the enzymatic activity of recombinant proteins if they are enzymes [1,12-13,21]. For proteins without known enzymatic activities, other functional assays are required. For example, TTP activity and its zinc dependency was evaluated by gel electrophoretic mobility shift assay (GMSA) according to its binding activity towards the AU – rich elements of the 3' – untranslated regions in mRNA molecules [2-3,10]. The effect of SDS on TTP activity and its binding kinetics for TNF – α mRNA AU – rich element was also evaluated by this method [2-4]. The capacity of purified recombinant TTP protein as a substrate for protein kinases was evaluated by in vitro phosphorylation assays [2-3,6].

3 Example: Expression and purification of anti-inflammatory protein tristetraprolin

Tristetraprolin (TTP) is an anti-inflammatory protein with the potential to be a therapeutic target for the prevention and treatment of inflammation-related diseases. Recent findings suggest that TTP plays an important role at the post – transcriptional level by binding to and destabilizing pro-inflammatory mRNAs such as tumor necrosis factor – α (TNF – α) mRNA [15]. The mRNAs encoding TNF – α and granulocyte – macrophage colony – stimulating factor (GM – CSF) are stabilized in TTP knockout mice and in cells derived from them [16,28]. Excessive secretion of these cytokines results in severe systemic inflammatory responses including arthritis, autoimmunity, and myeloid hyperplasia in the TTP knockout mice [29-30]. In contrast, up – regulation of TTP reduces inflammatory response in macrophages [31].

TTP is the prototype of proteins with two CCCH ($CX_8CX_5CX_3H$) zinc finger binding motifs separated by 18 amino acid residues [32-34] (Table 2). Similar zinc finger sequences are found in the GenBank database in a variety of species, ranging from human to yeast and plants (Table 3). TTP mRNA and protein are detected in a number of tissues including spleen, thymus, lymph node, lung, liver, and intestine [14,33]. Immunological, molecular, and proteomic studies have shown that TTP is a low – abundance, inducible, stable, cytosolic, and hyper – phosphorylated mRNA binding protein [10-14]. TTP is a highly phosphorylated protein [5,10,20,25] with extremely low – abundance in vivo [14], but it is highly inducible by a variety of factors such as lipopolysaccharide [14], insulin [36], cinnamon extract [37-39], and green tea extract [40-41].

表 2　人类、小鼠和老鼠 TTP 蛋白质氨基酸序列比较

Tab. 2　Comparison of amino acid sequences of TTP proteins from human, mouse, and rat

Human	MDLTAIYESLLSLSPDVPVPSDHGGTESSPGWGSSGPWSLSPSDSSPSGVTSRLPGR
Mouse	MDLSAIYESLQSMSHD. . LSSDHGGTES……LGGLWNIN. SDSIPSGVTSRLTGR
Rat	MDLSAIYESLMSMSHD. . LSPDHGGTES……SGGLWNINSSDSIPSGVTSRLTGR
Human	STSLVEGRSCGWV **PPPP**GFAPLAPRLGPELSPSPTSPTATSTTPSR **YKTELCRTFSE**
Mouse	STSLVEGRSCGWV **PPPP**GFAPLAPRPGPELSPSPTSPTATPTTSSR **YKTELCRTFSE**
Rat	STSLVEGRSCSWV **PPPP**GFAPLAPRPGPELSPSPTSPTATPTTSSR **YKTELCRTFSE**
Human	**SGRCRYGAKCQFAHGLGELRQANRHPKYKTELCHKFYLQGRCPYGSRCHFIH**NPSED
Mouse	**SGRCRYGAKCQFAHGLGELRQANRHPKYKTELCHKFYLQGRCPYGSRCHFIH**NPTED
Rat	**SGRCRYGAKCQFAHGPGELRQANRHPKYKTELCHKFYLQGRCPYGSRCHFIH**NPTED
Human	LAAPGHPPVLRQSISFSGLPSGRRTS **PPPP**GLAGPSLSSSSFSPSSS **PPPP**GDLPLS
Mouse	LALPGQPHVLRQSISFSGLPSGRRSS **PPPP**GFSGPSLSSCSFSPSSS **PPPP**GDLPLS
Rat	LALPGQPHVLRQSISFSGLPSGRRTS **PPPP**GFSGPSLSSCSFSPSSS **PPPP**GDLPLS
Human	PSAFSAAPGTPLARRDPTPVCCPSCRRA. TPISVWGPLGGLVRTPSVQSLGSDPDEY
Mouse	PSAFSAAPGTPVTRRDPNQACCPSCRRSTTPSTIWGPLGGLARSPSAHSLGSDPDDY
Rat	PSAFSAAPGTPVSRRDPTPACCPSCRRSTTPSTIWGPLGGLARSPSAHSLGSDPDDY
Human	ASSGSSLGGSDSPVFEAGVFAPPQPVAAPRRLPIFNRISVSE
Mouse	ASSGSSLGGSDSPVFEAGVFGPPQTPAPPRRLPIFNRISVSE
Rat	ASSGSSLGGSDSPVFEAGVFGPPQPPAPPRRLPIFNRISVSE

The three repeats of the four adjacent proline residues and the tandem CCCH zinc – finger domains were underlined in bold.

It was difficult to express and purify TTP from any expression system. In the earlier studies, TTP was expressed as a His – tagged protein in *E. coli* for antibody production and zinc – finger binding characterization [32,42]. TTP fused to GST was expressed in *E. coli* and used for in vitro phosphorylation study [30]. It was reported that those fusion proteins were extensively degraded and mostly precipitated [19 – 20]. We purified active TTP to homogeneity. TTP was expressed in *E. coli* as a MBP fusion protein. Soluble MBP – TTP fusion protein was partially purified by amylose resin affinity chromatography. TTP was further purified from the fusion protein by continuous – elution gel electrophoresis following protease digestion. The purified TTP free of MBP was shown to bind to TNF – α mRNA ARE probe.

3.1　Expression of recombinant fusion protein TTP in *E. coli*

TTP expressed in *E. coli* as a GST fusion protein were extensively degraded and mostly insoluble [19 – 20]. It has been reported that *E. coli* MBP promotes the solubility of polypeptides to which it is fused [24]. We therefore investigated the possibility of expressing and purifying recombinant TTP using MBP fusion system.

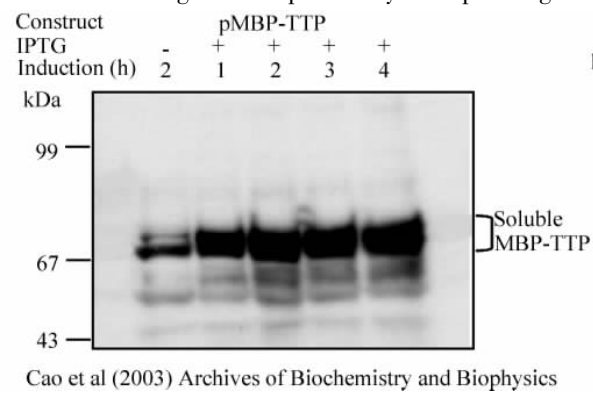


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图 2　在大肠杆菌中表达重组融合蛋白质 TTP

Fig. 2　Expression of recombinant fusion protein TTP in *E. coli*

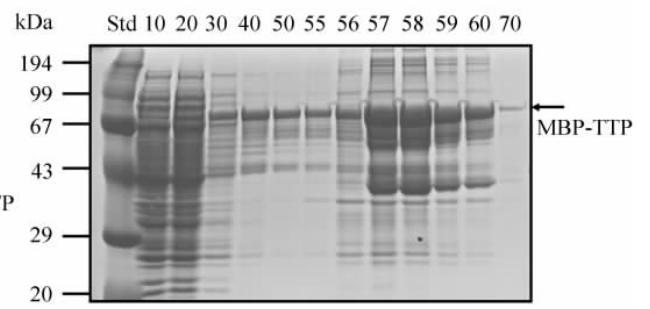


图 3　从大肠杆菌中纯化重组融合蛋白质 TTP

Fig. 3　Purification of recombinant fusion protein TTP from *E. coli*

表 3 不同物种中的串联 CCCH 锌指结合序列

Tab. 3 The tandem CCCH zinc finger binding domains in TTP and related proteins from various species

Species – Accession No.	N – Terminal Residue No.	Tandem CCCH Zinc Finger Binding Domains	C – Terminal Residue No.
Mammal and Frog CCCH Protein – TTP (ZFP36)			
Hs – M92843	103	YKTELCRTFSESGRCRYGAKCQFAHGLGELRQANRHPKYKTELCHKFYLQGRCPYGSRCHFIH	170
Mm – L42317	95	YKTELCRTYSESGRCRYGAKCQFAHGLGELRQANRHPKYKTELCHKFYLQGRCPYGSRCHFIH	171
Rn – P47973	96	YKTELCRTYSESGRCRYGAKCQFAHGPGELRQANRHPKYKTELCHKFYLQGRCPYGSRCHFIH	171
Rr – X63369	96	YKTELCRTYSESGRCRYGAKCQFAHGPGELRQANRHPKYKTELCHKFYLQGRCPYGSRCHFIH	171
Bt – P53781	101	YKTELCRTFSESGRCRYGAKCQFAHGLGELRQASRHPKYKTELCHKFYLQGRCPYGSRCHFIH	169
Xl – AAD24207	103	YKTELCRTFSETGTCKYGAKCQFAHGKIELREPNRHPKYKTELCHKFYLYGECPYGSRCNFIH	150
Mammal and Frog CCCH Protein – TIS11b (ZFP36L1)			
Hs – Q07352	104	YKTELCRPFEENGACKYGDKCQFAHGIHELRSLTRHPKYKTELCRTFHTIGFCPYGPRCHFIH	161
Mm – B39590	104	YKTELCRPFEENGACKYGDKCQFAHGIHELRSLTRHPKYKTELCRTFHTIGFCPYGPRCHFIH	161
Rn – NP_058868	104	YKTELCRPFEENGACKYGDKCQFAHGIHELRSLTRHPKYKTELCRTFHTIGFCPYGPRCHFIH	161
Xl – AAD24208	115	YKTELCRPFEENGSCKYGDKCQFAHGIHELRSLTRHPKYKTELCRTFHTIGFCPYGPRCHFIH	165
Mammal and Frog CCCH Protein – TIS11d (ZFP36L2)			
Hs – P47974	153	YKTELCRPFEESGTCKYGEKCQFAHGFHELRSLTRHPKYKTELCRTFHTIGFCPYGPRCHFIH	266
Mm – NP_008818	153	YKTELCRPFEESGTCKYGEKCQFAHGFHELRSLTRHPKYKTELCRTFHTIGFCPYGPRCHFIH	278
Rn – XM_238444	151	YKTELCRPFEESGTCKYGEKCQFAHGFHELRSLTRHPKYKTELCRTFHTIGFCPYGPRCHFIH	268
Xl – AAD24209	133	YKTELCRPFEENGACKYGEKCQFAHGFHELRSLTRHPKYKTELCRTFHTIGFCPYGPRCHFIH	154
Mammal CCCH Protein (No Human) – ZFP36L3			
Mm – XM_285657	122	YKTELCRPFEESGICKYGHKCQFAHGYRELRTLSRHPKYKTEPCRTFHSVGFCPYGTRCHFIH	540
Rn – XM_228661	121	YKTELCRPFEENGTCRYGNKCQFAHGYHELRTLSRHPKYKTEPCRTFHSIGYCPYGSRCHFIH	538
Yeast CCCH Protein			
Sc – NP_010435	204	YKTELCESFTIKGYCKYGNKCQFAHGLNELKFKKKSNNYRTKPCINWSKLGYCPYGKRCCFKH	48
Sc – NP_013237	170	YKTELCESFTLKGSCPYGSKCQFAHGLGELKVKKSCKNFRTKPCVNWEKLGYCPYGRRCCFKH	53
Sp – P47979	247	YKTEPCKNWQISGTCRYGSKCQFAHGNQELKEPPRHPKYKSERCRSFMMYGYCPYGLRCCFLH	15
Plant CCCH Protein			
At – NP_176853	232	MKTELCNKWQETGACCYGDNCQFAHGIDELRPVIRHPRYKTEVCRMMVTGAMCPYGHRCHFRH	15
At – NP_176987	232	TKTELCNKWQETGTCPYGDHCQFAHGIKELRPVIRHPRYKTEVCRMVLAGDNCPYGHRCHFRH	23
Os – BAB40156	182	FKTELCNKWEETGDCPYGDQCQFAHGVTELRPVIRHPRYKTAVCRMVLAGDVCPYGHRCHFRH	12
Worm, Fly, Fish, Oyster, and Frog CCCH Protein			
Cc – CAA71245	57	YKTELCSRYAETGTCKYAERCQFAHGLHDLHVPSRHPKYKTELCRTYHTAGYCVYGTRCLFVH	207
Ce – T21954	198	YKTELCRSWMDHGRCNYGERCQYAHGELEKRPVPRHPKYKTEACQSFHQSGYCPYGPRCHFIH	153
Ce – T21955	157	YKTELCRSWMDHGRCNYGERCQYAHGELEKRPVPRHPKYKTEACQSFHQSGYCPYGPRCHFIH	153
Cv – AAB69448	135	YKTELCRPFEESGHCKYGDKCQFAHGAHELRNLNRHPKYKTELCRTFHTIGFCPYGPRCHFIH	223
Dm – AAF48194	138	YKTELCRPFEEAGECKYGEKCQFAHGSHELRNVHRHPKYKTEYCRTFHSVGFCPYGPRCHFVH	248
Dm – CAA57066	136	YKTELCRPFEEAGECKYGEKCQFAHGSHELRNVHRHPKYKTEYCRTFHSVGFCPYGPRCHFVH	238
Dr – CAB55775	59	YKTELCSRYAETGTCKYAERCQFAHGLHDLHVPSRHPKYKTELCRTYHTAGYCVYGTRCLFVH	197
Xl – AAD24210	45	YKTELCSRYAESGFCAYRNRCQFAHGLSELRPPVQHPKYKTELCRSFHVLGTCNYGLRCLFIH	168
Consensus residue		**YKTELC**RPF E G **C**KYG K**C**QFA**H**G ELR . . RHPK **YKTELC**R F G **C**PYG R**C**HFI**H**	
		KSY D R A R Y D K. . . K RFRS V H W A V	
		TW H H Y Y L	

At (Arabidopsis), Bt (Bovine), Cc (Carp), Ce (C. elegans), Cv (Oyster), Dm (Drosophila), Dr (Zebrafish), Hs (Human), Mm (Mouse), Os (Rice), Rn (Norway rat), Rr (Black rat), Sc (Baker's yeast), Sp (Fission yeast), and Xl (Frog). The underlined residues within the sequence alignment represent the divergence from the consensus residues listed at the bottom row of the table. The consensus sequence of the tandem CCCH zinc finger domains is YKTELCX_8CX_5CX_3HX_{13}YKTEL–CX_8CX_5CX_3H.

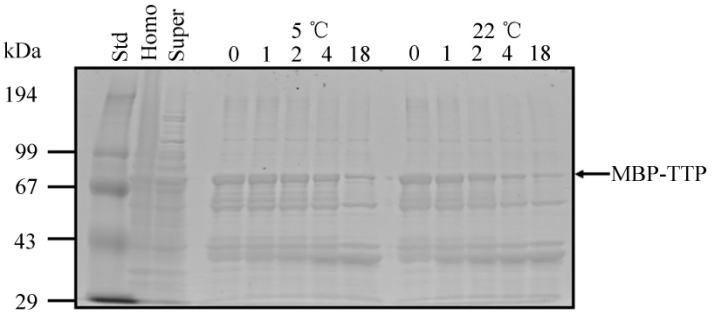
A PreScission protease recognition site was inserted between MBP and TTP for facilitating purification of TTP from the fusion protein. Immunoblotting analysis showed that MBP – TTP (calculated Mr. 75 265, pI 6.67) was degraded much less severe than GST–TTP in the over – expressed *E. coli* cells (Figure 2).

3.2 Purification of recombinant fusion protein TTP from *E. coli*

E. coli cells were homogenized by sonication. The 10 000 g – supernatant was applied onto an amylose resin column. MBP – TTP was bound to the amylose resin and eluted out with 10 mmol/L maltose. About 30 mg of total protein was eluted out from the column using cell extract from 1 – L culture. The majority of the eluted protein was MBP – TTP, although the eluted fractions also contained multiple other proteins (Figure 3). The identity of MBP – TTP in the eluted fractions was confirmed by immunoblotting using anti – MBP serum [2].

3.3 Protease digestion of recombinant fusion protein MBP – TTP

MBP – TTP eluted from amylose resin affinity column was digested with PreScission protease in a time – dependent manner (Figure 4). MBP – TTP digestion was detected following incubation with the protease for 1 h at 5 ℃ or room temperature. More than 80% of the full – length MBP – TTP was digested after 18 – h incubation at either temperature. Furthermore, PreScission protease was able to digest MBP – TTP following purification by Superose 12 size exclusion column and MonoQ anion exchange column (data not shown). The degree of protease digestion was similar in buffers with or without protease inhibitors including 1 mmol/L PMSF and 2 μmol/L leupeptin [2].



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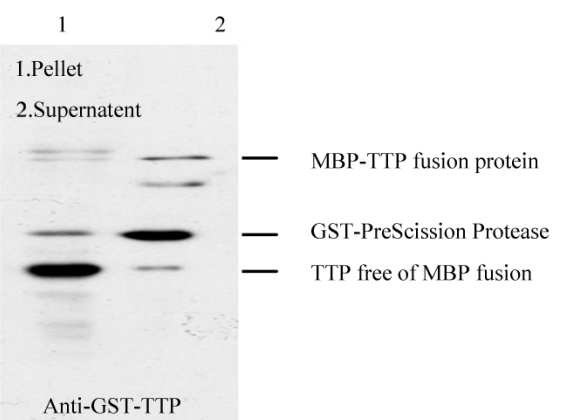
MBP – TTP eluted from amylose resin chromatography was digested with GST fusion PreScission protease at 5 ℃ or room temperature for 0, 1, 2, 4 and 18 h, separated along with the homogenate (Homo) and the 10 000g – supernatant (Super) by SDS – PAGE, and stained with Coomassie blue (Modified from [2]).

图 4　蛋白酶消化重组融合蛋白质 TTP

Fig. 4　Protease digestion of recombinant fusion protein MBP – TTP



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MBP – TTP was purified by amylose resin affinity column and digested with PreScission protease overnight. The digestion mixture was centrifuged at 20 000g for 5 min. Proteins in the fractions were separated by SDS – PAGE and stained by silver reagent (Modified from [2]).

图 5　蛋白酶消化后 TTP 沉淀

Fig. 5　Precipitation of recombinant protein TTP protein following PreScission protease digestion

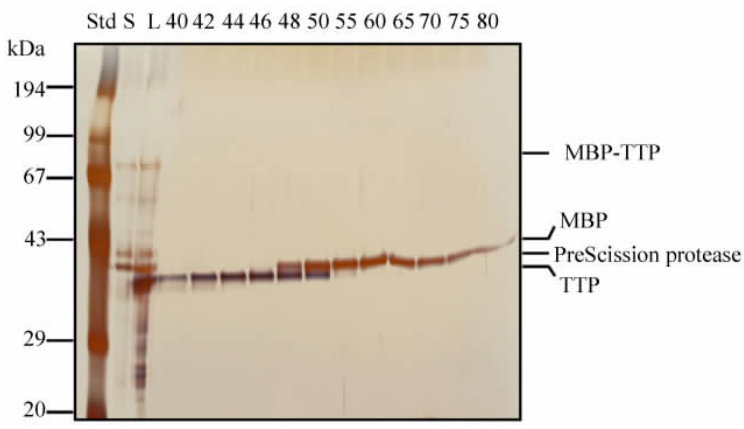
3.4 Difficulties in purification of recombinant protein TTP

Extensive efforts were directed to purify the released TTP from the digestion mixture by conventional chromatographic procedures, including MonoQ anion exchange, Superdex 75 and Superose 12 size exclusion. However, we were unable to purify TTP due to its extensive precipitation following protease digestion. For example, the protease digestion mixture was centrifuged at 20 000 g for 5 min and the supernatant was applied to a Superose 12 column or a Superdex 75 column (data not shown). The released TTP was largely detected in the pellet and not much TTP was recovered in the supernatant or in any of the fractions by silver staining or affinity – purified anti – GST – TTP antibodies (Figure 5). Part of theundigested

MBP – TTP was also precipitated in the pellet (Figure 5) . The identities of TTP (calculated Mr. 34 002 Da, pI 8.41) , MBP (calculated Mr. 41 258 Da, pI 4.91) ,and GST fusion PreScission protease (calculated Mr. 46 kDa) were determined by immunoblotting and MALDI – TOF mass spectrometry. TTP was conclusively determined to be the bottom band of the three bands about 40 kDa on SDS – PAGE [2] . Similar results were also obtained from MonoQ column (data not shown) . The precipitated TTP in the pellet was solubilized with 6 mol/L Guanidine – HCl and separated by Superdex 75. However,no TTP was found in any fraction by Coomassie blue or silver staining (data not shown) . We also solubilized TTP in the precipitated pellet with 0.1% trifluoroacetic acid and separated by HPLC using Sephasil C4 column. TTP was soluble in the buffer containing trifluoroacetic acid but both TTP and MBP were co – eluted in the same fractions for unknown reasons (data not shown) .

3.5 Purification of recombinant protein TTP by continuous – elution gel electrophoresis

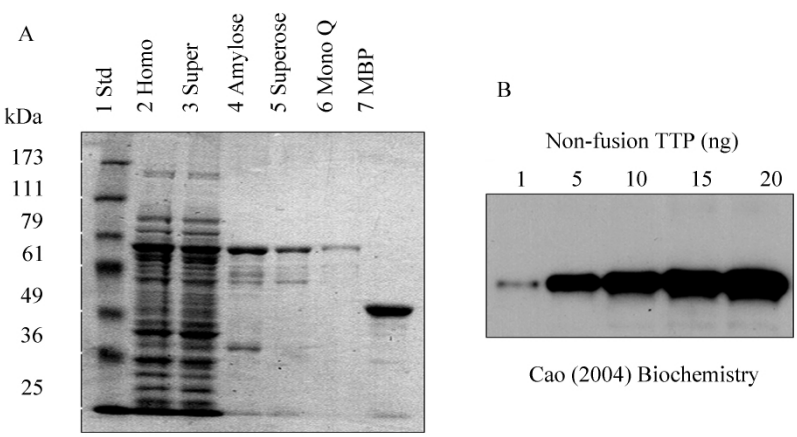
Since TTP was clearly separated from MBP on SDS – PAGE gel, we decided to purify TTP from the PreScission protease – digested mixture using continuous – elution gel electrophoresis technique (Figure 6) . MBP – TTP eluted from amylose resin column (Figure 3) was digested in digestion buffer containing amylose resin elution buffer, 20% glycerol, and PreScission protease at room temperature overnight. Proteins in the digestion mixture were separated by SDS – PAGE, eluted out of the gel, collected by fraction collector, and detected by silver staining and immunoblotting procedures. TTP was purified to homogeneity as shown by silver staining of proteins from fractions #40 – 46 (Figure 6) . Anti – GST – TTP antibodies recognized the undigested MBP – TTP, the PreScission protease,and the bottom



Proteins of overnight digestion were separated by SDS – PAGE, eluted continuously with SDS – PAGE running buffer, and collected with fraction collector. Proteins in the digestion and fractions were separated by SDS – PAGE and visualized by silver staining. Part of the eluted fractions was shown. S: supernatant from the digestion mixture showing poor recovery of digested TTP in the soluble fraction; L: the digestion mixture for CEGE separation (Modified from [2]) .

图 6 电泳纯化重组蛋白质 TTP

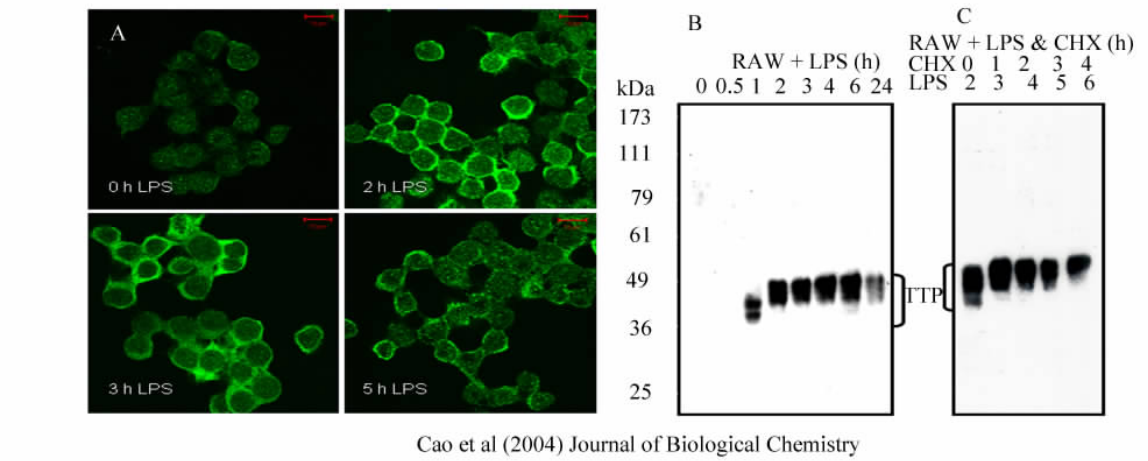
Fig. 6 Purification of recombinant protein TTP by continuous – elution gel electrophoresis



(A) Purification of MBP – TTP stained with Coomassie blue: The positions of MBP – TTP and MBP are indicated; (B) Detection limit of anti – MBP – TTP serum by western blotting. Non – fusion TTP purified from *E. coli* as (1, 5, 10, 15 and 20 ng) as indicated was probed with the anti – MBP – TTP serum (1: 10,000) for 1 h, GAR – HRP (1: 10,000) for 30 min, and exposed to X – ray film for 1 min (Modified from [2]) .

图 7 TTP 抗体生产

Fig. 7 Production of TTP antibodies



(A) TTP induction and localization in RAW 264.7 cells. RAW 264.7 cells were stimulated with LPS (0.1 g/mL) for 0, 2, 3 and 5 h as indicated, and stained with anti – MBP – TTP serum. (B) TTP induction in mouse cells. (C) TTP stability in RAW 264.7 cells. The cells were first stimulated with LPS for 2 h, followed by treatment with cycloheximide (CHX), an inhibitor of new protein synthesis, for the indicated times (Modified from [14]).

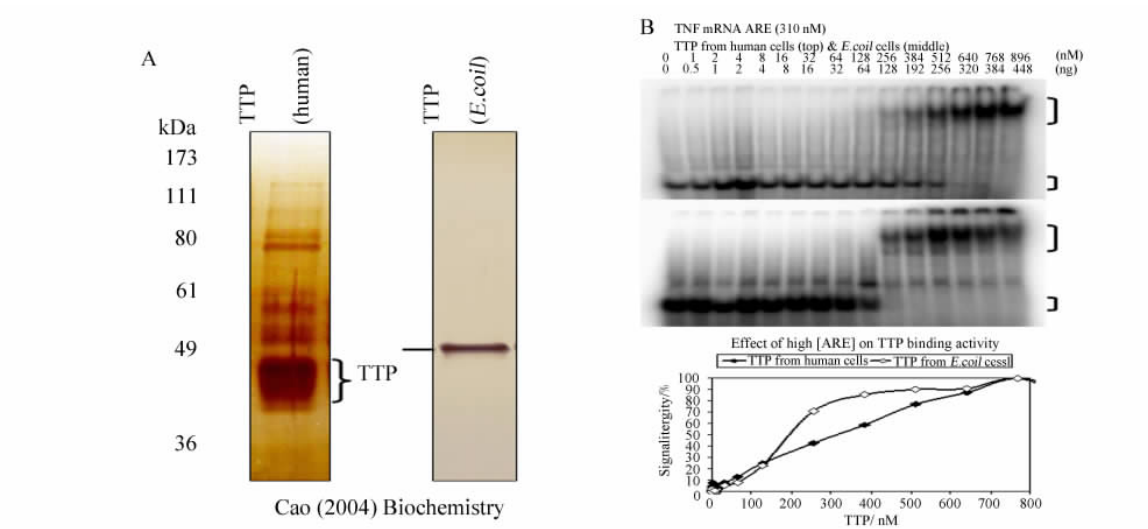
图 8　蛋白质 TTP 在小鼠细胞中的诱导, 定位和稳定性

Fig. 8　TTP induction, localization, and stability in mouse cells

TTP [2]. The identity of the purified TTP was determined by MALDI – TOF mass spectrometry and N – terminal sequencing. The first 13 amino acid residues of TTP were determined to be GPDLTAIYESLLS. The first two amino acid residues were derived from the PreScission protease site in the vector following PreScission protease digestion. The third sequenced residue was the second amino acid residue of the deduced human TTP amino acid sequence. These sequenced residues are in agreement with deduced amino acid residues and with the PreScission protease digestion site.

3.6　Utilization of recombinant protein TTP

The purified recombinant TTP was used for antibody production [3,14]. Anti – TTP sera can detect ng level of the protein [3] (Figure 7). These antibodies were used to analyze the expression, induction, and regulation



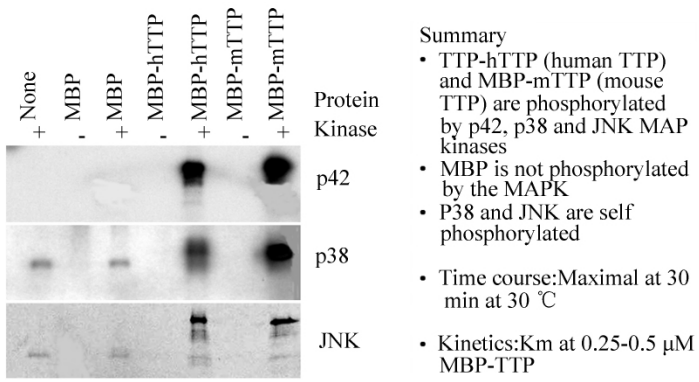
(A) Silver staining of TTP used for the binding kinetic studies. (B) ARE – binding activity using various concentrations of recombinant TTP purified from *E. coli* and human cells. Gels were exposed to Phosphorimager screens and the signal intensity of the TTP/probe complexes and free probes was analyzed with ImageQuant 5.1. The results represented the means of 2 – 4 duplications. The positions of TTP/probe complexes and free probes are indicated (Modified from [3,16]).

图 9　重组蛋白质 TTP – RNA 结合动力学

Fig. 9　Binding kinetics of recombinant TTP for TNF – α mRNA ARE

of TTP in endogenous cells and tissues. The antibodies helped to identify TTP as a low – abundance protein in normal tissues and cells, but TTP was massively induced by a number of factors, and the protein is localized in the cytosol in mouse and human cells [3,5,8,10,14,36 – 38] (Figure 8).

The purified recombinant TTP was also used for biochemical characterization. The activity of the purified recombinant TTP protein was evaluated by gel electrophoretic mobility shift assay [2 – 3]. The purified TTP bound to the TNF – α mRNA ARE probe in a time-and concentration–dependent manner [3] (Figure 9). Zinc is required for TTP activity since ARE binding activity of the purified TTP was significantly increased by 10 μmol/L $ZnCl_2$ but was almost completely inhibited by 500 μmol/L EDTA in the binding buffer [2 – 3].



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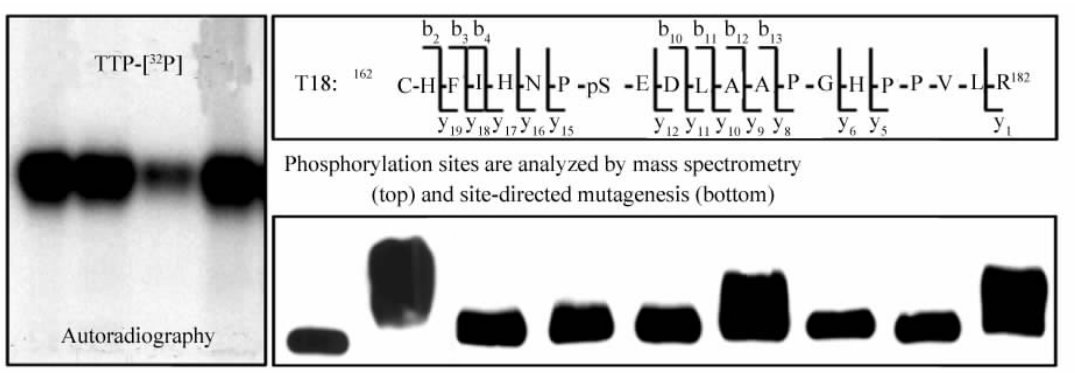
MBP – TTP was partially purified by amylose resin column and used as a substrate for p42, p38, or JNK MAP kinases. The reactions in were performed at 30 ℃ for 30 min (Modified from [2]).

图 10 体外重组蛋白质 TTP 磷酸化

Fig. 10 Phosphorylation of recombinant protein TTP *in vitro*

Recombinant TTP was phosphorylated by three members of the mitogen – activated protein kinase family, p42, p38, and JNK, with half – maximal phosphorylation occurring at approximately 0.5, 0.25, 0.25 μmol/L protein, respectively [2] (Figure 10). TTP was also phosphorylated in vitro by GSK3b, PKA, PKB, PKC, but not Cdc2 [5]. These results demonstrate that TTP is a substrate for a number of protein kinases in vitro and support the conclusion that TTP is highly phosphorylated at multiple sites in intact human and mouse cells [5,10,20,35,43,44] (Figure 11).



Cao (2004) Biochemistry; Cao et al (2006) Biochemical J & (2007) Expert Rev Proteomics

(Left) TTP is highly phosphorylated in transfected human cells [3]. Human embryonic kidney 293 cells were transfected with plasmid pHis – TTP. The cells were radio – labeled with ^{32}P – orthophosphate. Recombinant His – TP was affinity – purified by Ni – NTA beads. The proteins were separated by SDS – PAGE and detected by autoradiography. (Top right) TTP was phosphorylated at multiple sites in intact cells. Mass spectrometry analyses identified a number of phosphorylation sites in the recombinant His – TTP purified from transfected human cells [5]. (Bottom right) Site – directed mutagenesis identified several important amino acid residues which increased the electrophoretic mobility of the mutant TTP, implying mutation of the putative phosphorylation sites [10] (Modified from [3,5,10]).

图 11 体内重组蛋白质 TTP 磷酸化

Fig. 11 Phosphorylation of recombinant protein TTP *in vivo*

4 Conclusions

Recombinant protein expression and purification is one of the most important biotechnological methods. Production of sufficient amounts of recombinant proteins is normally required for antibody production, functional

assays, and structural determination. This paper highlights several approaches to increase recombinant protein production through selecting high level protein expression systems, optimizing protein expression conditions, minimizing protein purification steps, and maintaining protein structure and function. The information should help those who just start to learn the technology and for those who would like to increase the yield of their proteins of interest due to technical difficulties in the expression and purification of recombinant proteins.

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Abbreviations: DU1: maize starch synthase III Dull1 protein; GM – CSF: granulocyte – macrophage colony – stimulating factor; GMSA: gel electrophoretic mobility shift assay GST: glutathione – S – transferase; IPTG: isopropyl β – D – 1 – thiogalactopyranoside; MBP: maltose – binding protein; TNF: tumor necrosis factor; TTP: tristetraprolin; ZFP: zinc – finger protein.

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