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Establishment and optimization of a wheat germ cell-free protein synthesis system and its application in venom kallikrein

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ABSTRACT

Wheat germ cell-free protein synthesis systems have the potential to synthesize functional proteins safely and with high accuracy, but the poor energy supply and the instability of mRNA templates reduce the productivity of this system, which restricts its applications. In this report, phosphocreatine and pyruvate were added to the system to supply ATP as a secondary energy source. After comparing the protein yield, we found that phosphocreatine is more suitable for use in the wheat germ cell-free protein synthesis system. To stabilize the mRNA template, the plasmid vector, SP6 RNA polymerase, and Cu^{2+} were optimized, and a wheat germ cell-free protein synthesis system with high yield and speed was established. When plasmid vector (30 ng/μl), SP6 RNA polymerase (15 U), phosphocreatine (25 mM), and Cu^{2+} (5 mM) were added to the system and incubated at 26 °C for 16 h, the yield of venom kallikrein increased from 0.13 to 0.74 mg/ml. The specific activity of the recombinant protein was 1.3 U/mg, which is only slightly lower than the crude venom kallikrein (1.74 U/mg) due to the lack of the sugar chain. In this study, the yield of venom kallikrein was improved by optimizing the system, and a good foundation has been laid for industrial applications and for further studies.

Introduction

Cell-free protein synthesis systems have attracted considerable attention as a novel protein synthesis method, especially since continuous-flow cell-free protein synthesis was demonstrated [1]. Various cell-free protein synthesis systems derived from crude cell extracts, such as *Escherichia coli* extract, rabbit reticulocyte, lysate insect cell extract and wheat germ extract, have been used for many years as research tools in fundamental and applied biology. The *E. coli* extract was used in the ground-breaking experiments of Nirenberg and Matthaei [2], playing an important role in the discovery of the genetic code. Spirin and co-workers [1] reported a continuous methodology for cell-free translation, which was a major turning point in the development of cell-free protein synthesis technology. More recently, cell-free protein synthesis systems have shown remarkable utility in a number of protein synthesis technologies [3,4], including the production of pharmaceutical proteins [3,5–7] and their application in protein evolution and structural genomics [8,9].

One of the most important criteria for a protein production system is the ability to produce active proteins in their correct conformation. In this regard, wheat germ cell-free protein synthesis systems are superior to other cell-free protein synthesis systems. Wheat germ cell-free protein synthesis systems have the capability to produce high quality eukaryotic multi-domain proteins that outperform *E. coli*-based translation systems, regardless of whether the system is a cell-based recombinant system or a cell-free system. The polypeptide chains of eukaryotic multidomain proteins often elongate so quickly that the resulting proteins are often insoluble aggregates. Being derived from grain, the wheat germ cell-free system is free from biohazard and bioethical issues. Throughout the entire process, from producing the translation solution to synthesizing the proteins, bio-pollution is maintained at a minimum [10]. The wheat germ cell-free protein synthesis system allows the expression of proteins that would interfere with cell physiology. Therefore, the efficient production of toxic proteins, such as integral membrane proteins, can be achieved [11–14]. In addition, the cell-free system is an open system that can be supplemented with any necessary ingredient, such as phospholipids, detergents or molecular chaperones [15]. Moreover, cell-free transcription and translation systems can synthesize proteins with high speed and accuracy, approaching *in vivo* rates [16]. The implementation of the method also obviates the need for cell

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harvesting and cell lysis. Finally, PCR products can be expressed directly in the wheat germ cell-free synthesis system, which could greatly shorten the protein production time.

In our study, the venom kallikrein was used to study the wheat germ cell-free protein synthesis system. Venom kallikrein, which catalyzes the hydrolysis of kininogen and releases kinin, is a member of the serine protease family [17]. Venom kallikrein hydrolyzes kininogen, which can increase vasodilation much more efficiently than other proteases and has been widely used for many applications, such as for treating hypertension. The enzyme has attracted considerable attention due to its therapeutic potential for myocardial infarctions and thrombotic diseases. The enzyme acts on fibrinogen to lead to defibrinogenation of blood, which results in a decrease in blood viscosity [17–19]. Venom kallikrein is mainly extracted from snakes via a complicated operation that is high in cost. The toxic substance in the snake venom is dangerous to humans. Therefore, the establishment of a recombinant system to produce venom kallikrein is necessary. An *E. coli* recombinant system has been used to produce venom kallikrein for biochemical analysis in our laboratory, but it remains difficult to generate a supply of the protein with desirable quality and activity. An alternative approach based on wheat germ cell-free (CF)[1] translation has therefore been adopted for the production of venom kallikrein. The capability to produce high quality eukaryotic multi-domain proteins guarantees the activity and efficacy of the recombinant venom kallikrein. Most importantly, the safety of the system ensures that the recombinant protein is safe for human beings.

Although we identified many advantages of wheat germ cell-free synthesis system for synthesizing proteins, there were also several disadvantages. First, there is a limited energy of the system even in the presence of regenerating ATP, and the instability of the mRNA templates restricts the protein yield. Second, although the conformation of proteins that are produced in the system could be accurate, the lack of posttranslational modifications and glycation may affect the characterization of functional proteins. The first disadvantage is the primary obstacle of industrialized production. Several measures were taken to overcome this obstacle, including the selection of a secondary energy material, the addition of Cu^{2+}, the supply of the expression plasmid and SP6 RNA polymerase, and the adjustment of temperature. Following these procedures, venom kallikrein was first produced with the optimized wheat germ cell-free protein synthesis system at a higher yield. Then, the recombinant venom kallikrein was purified and compared with the crude kallikrein.

Materials and methods

Vectors and materials

The vector, pCS^{2+}, which was used for protein expression, was kindly supplied by Professor Jian Zhang Institute of the Genetics and Developmental Biology, Chinese Academy of Sciences. *Lunxuan 987* wheat was purchased from the Beijing Shunxin agriculture corporation. Both crude venom kallikrein and crude venom kallikrein lacking a sugar chain were supplied by Saisheng Pharmaceutical Company in Beijing. All other chemicals were of analytical grade and obtained from commercial sources. Venom kallikrein gene has been cloned and preserved in our laboratory.

DNA cloning

Several reports describing the cell-free expression of PCR products have already been published [20,21]. However, in our study, the constructed plasmid was chosen as a template to transcribe mRNA because the PCR products can be degraded rapidly by the exonucleases present in the cell-free extract. The plasmid which could exist for a longer amount of time and produce more mRNA must be more stable and be able to be obtained more easily in high quantity than the PCR products.

The venom kallikrein gene was cloned, with a His-tag including 6 histidines at the N-terminal position into the pCS^{2+} vector. This expression plasmid was based on the SP6 transcription system. In the beginning, PCR was conducted with one pair of primers (forward primer: 5′-CGCGGATCCATG*CACCATCATCATCATCAT*GTC ATTGGAGGTGATGAATGT-3; reverse primer: 5′-CCGCTCGAGTCACGGGGG GCATGTCAC-3′). However, we have obtained few PCR products because the sequence of His tag (italics) is too long. So three primers were designed (forward primer 1:5′- TCATCATCATCATGTCATTGGAGGTGATGAATGTAACA-3′; forward primer 2: 5′-CGCGGATCCATG*CACCATCATCATCATCAT*GTCATTG GAGG-3′; reverse primer: 5′-CCGCTCGAGTCACGGGGGGCATGTCAC-3′). PCR reactions were conducted for two times with the forward primer 1 and reverse primer for the first time and then with the forward primer 2 and reverse primer for the second time. The BamHI and XhoI restriction sites were designed into the forward primer 2 and reverse primer, respectively. The programs of two PCR reactions were the same and conducted in DNA ENGINE Peltier PCR machine (Bio-Rad). After initial denaturation at 94 °C for 5 min, the PCR reaction was carried out using Pyrobest Taq polymerase. The conditions for each cycle were as follows: denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s, and extension at 72 °C for 1.5 min. A final extension step for 10 min at 72 °C was added at the end of the 35 cycles. Amplified PCR products were ligated into pGEM-T Easy vector (Promega, USA) and the resultant recombinant plasmids were sequenced. Then the correct plasmid was digested with BamHI and XhoI and ligated into pCS^{2+} with the same enzymic sites, generating the expression plasmid. The Gel Extraction Kit (CWBio) was used to extract PCR products from gel. The expression plasmid was transformed into the *E. coli* DH5α and identified by restriction analysis and sequencing.

SDS–PAGE and western blotting

Standard SDS–PAGE gel electrophoresis [22] was performed on 15% polyacrylamide gels before using Coomassie blue stain. For the Western blots, proteins were transferred after migration in a polyacrylamide gel to a nitrocellulose membrane (Immobilon-P Transfer Membrane, nitrocellulose membrane, 0.45 mm, Millipore) using a Trans-Blot wet transfer cell (Bio-Rad) for 90 min at 80 V and a 5.5 mA/cm^2 intensity for one minigel. The nitrocellulose membrane was then saturated with BSA (5%) in TBS–Tween (Tris–HCl 25 mM pH 7.6, NaCl 0.15 M, Tween 0.05%). Incubation was performed with the primary antibody directed against the His-tag (CWBio) domain for venom kallikrein, prior to incubation with the secondary antibody (anti-mouse IgG, AP conjugate, CWBio). The labeled proteins were revealed using the 5-bromo-4-chloro-3-indolyl phosphate/tetranitroblue tetrazolium chloride (BCIP/NBT Zhongshan Goldenbridge Biotechnology).

Isolation of wheat embryos and extract preparation

The preparation of the extract was based on the protocol described by Endo and coworkers [23,24]. We used biologically cultured and untreated durum wheat *Lunxuan 987* seeds (*Triticum durum desf.*) to prepare the wheat germ extracts. The embryos were extracted by hand. Only perfectly intact and clean embryos were preserved. To prepare the extract, 10 g of wheat embryos were used. All steps during the extract preparation were carried out rapidly and in a cool and clean environment. To clean the

[1] *Abbreviations used:* CF, cell-free; PEP, phosphoenol pyruvate ; BAEE, N-benzoyl-L-arginine ethyl ester hydrochloride; CK, creatine kinase; Pox, pyruvate oxidase; RKV, recombinant venom kallikrein.