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Research progress of limulus coagulation mechanism and limulus reagents

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Abstract: The coagulation system of Limulus is mainly composed of coagulation proteins such as factor G, factor C, factor B, and prothrombin. The traditional horseshoe crab reagent for endotoxin detection is not specific, and its colorimetric reaction is activated through two pathways: one is activated by endotoxin to activate factor C, and the other is activated by (1,3)- β-D-glucan activates factor G. The horseshoe crab reagent is composed of a series of serine proteases and is a biological reagent made by treating horseshoe crab blood cell lysate. The detection principle is that the Limulus amebocyte lysate clotting factor protein is activated by endotoxin or fungal glucan to generate a series of cascade reactions to form insoluble gel substances, and then it is used to detect bacterial endotoxin or fungal glucan contaminated in biological products through the chromogenic reaction of the chromogenic matrix. Mainly used in multiple fields such as drug inspection, food, medical equipment, clinical medical research, etc. This article mainly summarizes and summarizes the coagulation mechanism of horseshoe crab and the current development status of horseshoe crab reagents.

1. Introduction

The innate immune system of horseshoe[1] crab relies entirely on a unique and highly effective host defense system and coagulation system. The coagulation cascade reaction of the horseshoe crab coagulation system is mainly composed of factors C, G, B, and prothrombin, as well as a type of prothrombin. These active substances can be detected by trace amounts of bacterial endotoxins or (1,3)-β- D-glucan is activated to produce gel reaction. This coagulation cascade reaction is crucial for preventing lymphatic leakage and immobilizing invading microorganisms, and is an important defense system for horseshoe crab to resist exogenous pathogens.

The horseshoe crab is a second level protected animal in China. In July 2019, the Chinese horseshoe crab was listed on the IUCN Red List - Endangered (EN). The growth cycle of horseshoe crabs is very long, and it takes nearly 13 years to complete reproduction. Due to the impact of some human activities such as land reclamation, mudflat development, overfishing and so on, the resources of horseshoe crab (Limulus amebocyte lysate) have been sharply reduced. Therefore, it has broad development prospects to replace the clottin factor G isolated from the hemolytic lysates of Limulus amebocyte (Limulus amebocyte lysate) by recombinant Limulus amebocyte clottin factor G.

2. Tachypleus

Horseshoe crabs have a long history and are known as "living fossils". Now only has four kinds of horseshoe crabs in the world, are round tail king crab, king crab, the south of China horseshoe crabs and America horseshoe crabs. The body surface of the horseshoe horseshoe is covered with a chitin exoskeleton and is dark brown in color. With a well-developed horseshoe shaped dorsal armour on its head and chest, it is often called a horseshoe crab. The body is nearly ladylike, divided into head chest, abdomen and tail three parts, male and female adults often together.

In February 2019, sequencing, assembly and annotation of the horseshoe crabs, in order to understand the characteristics of the genomes of living fossils and help scientists to protect this endangered species. The resulting total genome size is 1.943 GB, which is estimated to cover 90.23% of the estimated genome size. And the transcriptomes of three larval stages were constructed to study candidate genes associated with larval development and to verify the validity[2] of the annotations. In order to further study its unique immune system, the genome was assembled with a nanopore reader in the same year to obtain a reference genome size of 2.16 GB, and a high-quality chromosomal level horseshoe crab genome and unique genomic features[3] were constructed. In 2020, the Chinese horseshoe crabs genome will build its evolution picture and developed the immune system[4]. The formation of new isomers, new genetic loci, fusion isomers and transcriptome structure were studied by the

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third generation sequencing and transcriptome sequencing techniques to further reveal the complexity[5] of the transcriptome of Limulus sinensis.

2.1. The clotting mechanism of Limulus limulus

The lysate of horseshoe crab blood cells contains two coagulation mechanisms as shown in Figure 1. One is a coagulation cascade reaction that can be activated by trace amounts of bacterial endotoxins (LPS), consisting of three coagulation proteins: Factor C (FC), Factor B (FB), and Proclotting Enzyme (PCE), as well as one coagulation protein. LPS activates FC, and the activated FC further activates FB. The activated FB then converts PCE into Clotting Enzyme (CE), and CE breaks down into clotting protein to form insoluble gel. Another coagulation mechanism is through (1,3)- β- D-glucan activates Factor G (FG), and the activated FG converts PCE into CE, which splits the coagulated protein to form insoluble gel.

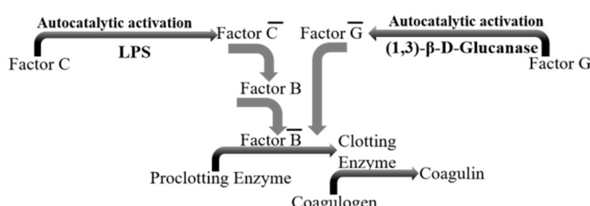

Fig. 1. Schematic diagram of the clotting mechanism of Limulus limulus.

Endotoxins, also called lipopolysaccharides, are components of the cell wall of Gram-negative bacteria. Endotoxins are the most well-studied biological pyrogen. They are ubiquitous and not easily inactivated. When they enter the body, they can cause severe pyrogen reactions in patients, leading to fever, shock and even death. The control of endotoxin is a challenge that major pharmaceutical companies and medical device industry must face. Sensitive and reliable quantitative detection technology of endotoxin is very important.

The composition and structure of endotoxin in different strains are different. The general structure of endotoxin consists of three parts: O antigen (specific O chain), core polysaccharide and lipid A. Among them, the specific O-chain has high antigenicity, and the O-chain polysaccharide is different in different bacteria with high specificity. The A part of lipid has high toxicity. The structure of lipid A is basically similar among different Gram-negative bacteria. Therefore, all infections caused by Gram-negative bacteria, although different strains, have roughly the same toxic effects caused by endotoxins. The molecular structure of endotoxins is shown in Figure 2.

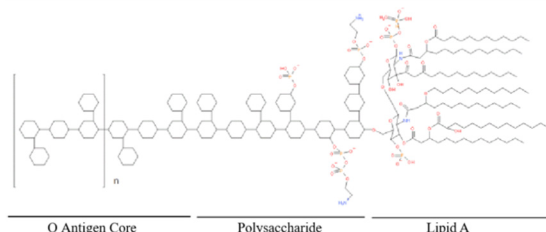

Fig. 2. Molecular structure of endotoxin.

It has been found that endotoxin activates the enzyme-enzyme cascade process in limulus amebocyte lysate (LAL) to form proteolytic enzyme, coagulase. Coagulase in turn hydrolyzes the coagulated proteogen at the Arg-Lys and Arg-Gly junctions, allowing the release of lactone C chains. When the peptide C chain is released, the structure of the coagulated proteogen collapses and forms a gel. There are several synthetic substrates on the market that mimic the site of action of clottin factor G, all of which contain the -Gly(Ala) -ARG-P-nitroaniline (PNA) chain. Coagulase hydrolyzes the synthetic substrate and releases the free PNA, which is read at 405 nm beam photometer. The amount of vibration of the PNA is proportional to the amount of endotoxin in the sample. A completely automated method[6] for hair color substrate LAL analysis by robotic system was constructed.

In the absence of endotoxin, very low concentrations of carboxymethylated (1,3)-beta-D-glucan, a water-soluble anti-tumor polycystic, can activate prothrombin by a different mechanism than that activated by endotoxin, causing the gelation of amoeba cell lysate from horseshoe crabs and inducing amylation activity of limulus thrombin, Thus further demonstrating that the mechanism by which (1,3)-β-d-glucan activates prothrombin is different from that activated by endotoxin, and identifying the presence of certain (1,3)-β-d-glucan-sensitive components in the lysate as factor G.

2.2. Horseshoe crab coagulation factor

2.2.1 Factor C

Factor C[7] is an endotoxin-sensitive intracellular serine proteasogen that initiates the clotting cascade in the limulus hemolymph system. Factor C isolated from the lysate of natural limulus cells is a single chain glycoprotein with a molecular weight of 123 kD or a double chain consisting of a heavy 80 kD chain at the N-terminal of the factor C and a light 43 kD chain at the C-terminal of the factor C. Under the action of bacterial endotoxin, it is automatically catalyzed into active factor C. After the activation of factor C, the Ph-Ile bond in the light chain is broken, and the molecular weights of A and B chains are 79 kD and 34 kD, respectively. B chain has a trace of amino protease region, and the binding site of endotoxin is located in the heavy chain. The SUSHI region in the N end of the heavy chain is the key site for binding with endotoxin. Factor C, usually in the form of zymogen, can be activated by trace amounts of bacterial endotoxin to produce a gel reaction and is the promoter of the clotting cascade. In the body, factor C is a perfect biosensor that alerts horseshoe crabs to the presence of gram-negative invaders. Trap the invader at the end of the hemostasis, killing it and limiting further infection. The domain of the factor C is shown in Figure 3.

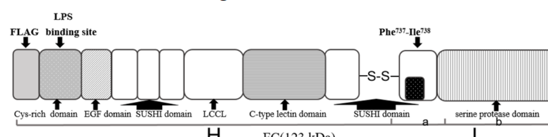

Fig. 3. Factor C domain.

2.2.2 factor G

factor G is an intracellular serine proteasogen that differs from other known serine proteasogen in that factor G consists of two distinct subunits alpha (72 kDa) and beta (37 kDa) that are autocatalytically converted into active factors in the presence of (1,3) -beta-d-glucan. The factor G can be activated by trace amounts of (1,3) -β-D-glucan to produce a gel reaction. The domain of the factor G is shown in Figure 4.

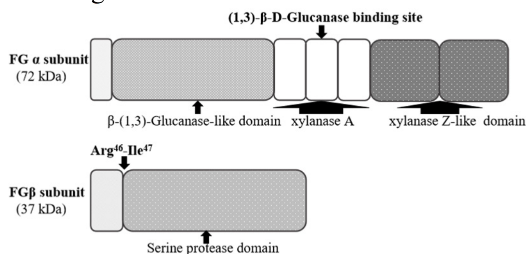

Fig. 4. Domain of Factor G.

2.2.3 factor B

Intracellular coagulation factor B, which is closely related to the hemolymph coagulation system of horseshoe crabs. The researchers found that without 2-mercaptoethanol, one band (Mw=64000) was obtained on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified preparation, while three bands (Mw=64000, 40000 and 25000) were detected on the reduced SDS-PAGE. This preparation is composed of clotting factor C, which is converted into an activated form by factor B. Mw=56000 is composed of a disulfide bridged heavy chain (Mw=32000) and a light chain (Mw=25000). Recombinant studies using purified factor C, factor B, prothrombin, and prothrombin in the presence of lipopolysaccharide have shown that factor B is necessary to complete the sequential activation of the lipopolysin system, and that it specifically activates prothrombin to active thrombin.The domain of the factor B is shown in Figure 5.

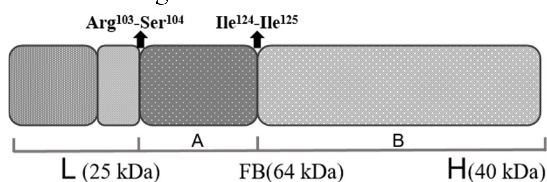

Fig. 5. Domain of Factor B.

2.2.4 Coagulase

Procoagulase is an intracellular serine proteinogen closely related to endotoxin-sensitive limulus hemlymphatic coagulation system. Procoagulase plays an important role in the formation of gels by activation of factor C and factor G.The domain of the Procoagulase is shown in Figure 6.

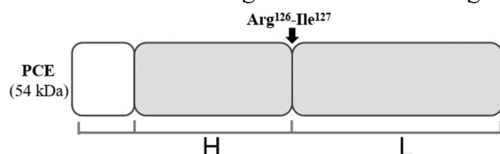

Fig. 6. Domain of Procoagulase.

2.2.5 Coagulating proteinogen

Coagulogen in limulus may be alkaline polypeptide chain, and its primary structure includes A chain, B chain and C peptide. There are active tripeptides in the A chain and the C peptide near the C end. When the coagulogen is activated by coagulase to coagulate protein, it breaks at the arginine of the tripeptide, and the peptide is released to form the coagulable protein gel molecule. Limulus coagulogen is a key part of the coagulation system of limulus lysate. The crystallographic diffraction structure of coagulagen is shown in Figure 7.

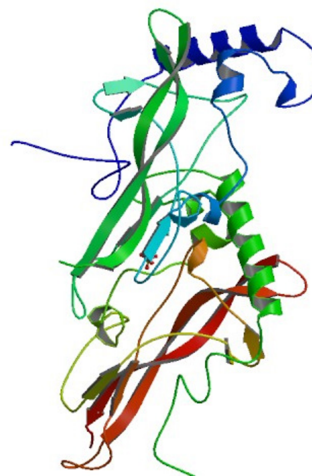

Fig. 7. Diffraction structure of solidified proteogen crystal.

2.3. Differential blocking of coagulation activation pathway of horseshoe crab blood cell lysate

The clotting system of limulus lysate (LAL) can be activated by two pathways, one is the endotoxin regulatory pathway and the other is the (1,3) -beta-D-glucan regulatory pathway. In the limulus enzyme-linked immunosorbent assay (ELISA), the endotoxin-activated pathway can be inhibited differently-by two methods. The reactivity of LAL to 10 different endotoxin preparations can be suppressed hundredfold by the former method, either by combining DSO with polymyxin B or by monoclonal antibodies against limulus factor C. And can be almost completely blocked by the latter method, regardless of the source of the endotoxin[8].

A liulus intracellular coagulation inhibitor, named Lici, isolated from blood cells of Japanese horseshoe crabs, is a single chain glycoprotein with an estimated apparent M(R)=48,000 by SDS-Polyacrylamide gel electrophoresis. It blocks the amylolytic activity of lipopolysaccharide-sensitive serine protease factor C by forming a 1:1 covalent complex with protease.

In ongoing studies on limulus serine proteinases, we have identified another inhibitor, Lici type-2(LICI-2), which inhibits not only factor C, but also limulus thrombin. In our ongoing studies on limulus, we have found another inhibitor, named Lici Type 3 (LICI-3), which strongly inhibits the (1,3) -beta-d-glucan-sensitive serine protease, factor G.

3. limulus agents

3.1. Introduction of limulus agents

Limulus agents consists of a series of serine proteases. Limulus lysate is a biological reagent prepared by processing limulus hemocyte lysate. Limulus lysate can be triggered by trace amounts of endotoxin or mycoglucan, and eventually form a gel at the end of the reaction[9]. It can be used to detect contaminated bacterial endotoxin or mycoglucan in biological products. Limulus is widely used in many fields such as drug testing, food, medical equipment, clinical medical research and so on. The types of limulus lysate are shown in Table 1.

Table 1. Types of limulus agents.

Classification Method	Species of Limulus	Characteristics
Sources	Chinese limulus agent	The raw material is the blood of Chinese horseshoe crabs
	American limulus agent	The raw material is the blood of the American horseshoe horseshoe
Test methods	Gelated limulus reagent	Judge the result by whether or not a gel is produced; Qualitative or semi-quantitative test, the earliest limulus reagent used at home and abroad.
	Dynamic turbidimet ric limulus reagent	Determination of the content based on the change in turbidity that produced the gel.
	Dynamic chromoge nic limulus reagent	According to the change of the color of the substrate after the reaction, the content of the substance to be measured is judged, and the terminator is not necessary in the reaction process.
	End color developme nt method limulus reagent	According to the change of the color of the substrate after the reaction, the content of the substance to be measured is judged, and the terminator needs to be added in the reaction process.
Usage characteristics	Common limulus reagent	A sample for testing endotoxin limits.
	High sensitivity limulus reagent	Suitable for testing samples with low endotoxin limit.
	Specific limulus agent	It is suitable for samples with complex composition and interference with limulus.
	Quantitati ve limulus reagents	Suitable for quantitative determination of samples

3.2. Development process of limulus agent

The study of limulus reagent was first developed by Levin and Bang in 1968. Based on their observation of trace endotoxin coagulant lysate of limulus blood cells, limulus hemocyte deform lysate (LAL) was extracted in the United States to make limulus reagent for the detection of trace endotoxin, and was approved by the US FDA. In the mid-1970s, Nakamura purified limulus factor B from LAL, and found factor C when further purifying the factor B, which revealed the molecular mechanism of LAL's determination of endotoxin. In the 1980s, Japan introduced specific limulus reagents that only reacted with endotoxins or fungal polysaccharides. This preparation process was difficult and the cost of limulus reagents was high, so it was difficult to popularize and apply. In 1983, it was proposed that magnesium could improve the sensitivity of endotoxin detection. In 1984, the method of detecting endotoxin by using hair color substrate was developed, and the limulus agent was further improved. In 1985, it was found that the lysate of limulus cells could be gelated by a very low concentration of (1,3)-β-D-glucan (CMPS) in the process of limulus reagent detection, and the limulus reagent specific detection was developed. From the late 1980s to the early 1990s, the clottin factor G in limulus blood were isolated and purified one after another. In 1991, the sensitivity of limulus lysate to (1,3)-β-d-glucan was further studied. In 1994, it was revealed that the endotoxin clotting pathway could be blocked by the combination of dimethyl sulfoxide and polymyxin B, or by monoclonal antibodies against factor C, which facilitated the development of limulus. In 1995, mass production of Tachypleus lysate began in China. Japan has long been commercialized, and has entered the practical stage, in China is still in the research and development, trial stage. In 1997, the quality of "New North" limulus reagent reached the international level, and it was widely used and sold to the vast areas of China, the United States and Japan. In 2005, Limulus limulus reagent was extracted from biological active ingredients in limulus blood to prepare specific limulus reagent, which reached the advanced level of similar technology in China and filled the gap of specific limulus reagent industrialization project in Guangxi region. In 2010, the method of detecting fungal glucan with specific limulus lysate in China, which was not described in detail in domestic literature, reached the leading level in China. In 2012, the "Fungal (1,3)-β-D-glucan detection kit" developed by a Guangdong company broke the monopoly of the United States in this technical field.

3.3. Limulus industry status

At present, limulus[10] blood is the main raw material for producing limulus reagents. However, with the development of limulus industry technology, the demand for limulus in various industries has increased, and the excessive consumption of limulus resources has made it difficult for natural limulus blood to supply a large number of limulus reagents. The lack of raw materials will directly lead to the stagnation of the limulus industry. At the same time, the application field of the traditional limulus lysate is limited due to the difference in sensitivity and poor specificity.

Some domestic researchers have cloned and expressed limulus factor C and G factor in E. coli, and obtained recombinant limulus factor C that can be activated by endotoxin and recombinant G factor that is sensitive to (1,3)-β-d-glucan. The use of recombinant factor C in the detection of endotoxin has not been reported in China. The development level of limulus lysate in foreign countries is higher than that in China. The quality and standard of limulus produced in the United States are the highest, the output is the largest, and the technical content of the products is also the highest. Although the specific limulus lysate has been successfully prepared by gene cloning and heterologous protein expression abroad, compared with the natural limulus lysate, the recombinant limulus lysate for the detection of endotoxins still has some problems such as low sensitivity, high production cost and different detection results[11]. The addition of anti-lipopolysaccharide to the lipopolysaccharide lysate could not completely block the interference of endotoxin to the lipopolysaccharide lysate test.

4. Summary

As endotoxins and (1,3)- β- In vitro testing tool for d-glucan, endotoxin on edges or (1,3)- β- The changes in sensitivity and specificity of d-glucan, as well as the decrease in peripheral blood supply, pose increasing challenges to the peripheral industry. This requires continuous exploration and improvement of methods for detecting endotoxins and (1,3) - d-glucan, and it is also hoped that there will be new breakthroughs in the research field of recombinant horseshoe crab reagents.

References

1. Lu C C. (2021) The impact and countermeasures of the adjustment of the National Key Protected Wildlife List. J. Chinese Fisheries, (06): 43-45. https//www.cnki.net.
2. Liao YY, Xu PW, Kwan KY. (2019). Draft genomic and transcriptome resources for marine chelicerate Tachypleus tridentatus. J. Sci Data, 6:19 0029. Doi: 10.1038 / sdata. 2019.29
3. Gong L, Fan G, Ren Y. (2019). Chromosomal level reference genome of Tachypleus tridentatus provides insights into evolution and adaptation of horseshoe crabs. J. Mol Ecol Resour, 19(3): 744-756. doi:10.1111/1755-0998.12988.
4. Zhou Y, Liang Y, Yan Q. (2020). The draft genome of horseshoe crab Tachypleus tridentatus reveals its evolutionary scenario and well-developed innate immunity. J BMC Genomics,21(1):137. doi:10.1186/s12864-020-6488-1.
5. Lou F, Song N, Han Z, Gao T. (2020). Single-molecule real-time (SMRT) sequencing facilitates Tachypleus tridentatus genome annotation. J. Biol Macromol, 147:89-97. Doi: 10.1016 / j.i jbiomac. 2020.01.029.
6. Tidswell EC. (2023). A Nontrivial Analysis of Patient Safety Risk from Parenteral Drug and Medical Device-Borne Endotoxin. J. Drugs R D, 23(1):65-76. doi:10.1007/s40268-023-00412-yMiao
7. Kawabata SI, Shibata T. (2022). New insights into the hemolymph coagulation cascade of horseshoe crabs initiated by autocatalytic activation of a lipopolysaccharide-sensitive zymogen. J. Dev Comp Immunol, 135:104491. doi:10.1016/j.dci.2022.104491
8. Baker E, Ponder J, Oberdorfer J. (2023). Barriers to the Use of Recombinant Bacterial Endotoxins Test Methods in Parenteral Drug, Vaccine and Device Safety Testing. J. Altern Lab Anim. 2023;51(6):401-410. doi:10.1177/02611929231204782
9. Pei YS, Cai T, Chen C. (2022). Application study of supplementary methods for bacterial endotoxin testing in the 2020 edition of the Chinese Pharmacopoeia [J]. Chinese Journal of Modern Applied Pharmacy, 2022, 39 (06): 822-826. DOI: 10.13748/j.cnki. issn1007-7693.2022-06.019
10. Maloney T, Phelan R, Simmons N. (2018). Saving the horseshoe crab: A synthetic alternative to horseshoe crab blood for endotoxin detection. J. PLoS Biol, 16(10): e2006607. doi:10.1371/journal.pbio.2006607
11. Mohamed H, Burroughs S, Summerville S. (2022). Inactivation of bacterial endotoxin in water using a pressurized microfluidic ultra-high temperature (UHT) pasteurizer. J. Journal of Water Process Engineering, 48: 1-5. DOI:10.1016/j.jwpe.2022.102927.