CHAPTER IV

DISCUSSION

Most of the horseshoe crabs were obtained from Samutphrakarn and four lots each were obtained from Chonburi and Samutsakorn. The preparation of amoebocyte lysate was preliminary carried out in eleven small lots to evaluate conditions and reagents to be used for protocol preparation. The preliminary experiments were conducted by varying anti-clotting solution and method of lysis. 0.125% (P-01) and 0.05 M (P-03 and P-04) of EDTA could not give firm gelation with endotoxin even in the level of 100 ng/mL. The 0.125% EDTA was too little to separate amoebocytes and stabilize their cell membrane from eruption which caused clumping of amoebocytes. The lysis of cells at this stage would leave only small amount of cells for the next step of preparation. The 0.05 M EDTA (P-03, P-04) was enough to separate amoebocytes and stabilize their cell membrane, but it might associate with cell membrane and incorporated in lysate. The trace of EDTA in lysate inhibited the gelation reaction as stated by Niwa et al(73). The 0.025 M EDTA (P-02, P-04 to P-11) was enough to separate amoebocytes and stabilized their cell membrane, and might be washed out completely during washing process. Therefore 0.025 M EDTA was used as anti-clotting solution.

The lysis method performed in P-02 and P-04 by using Tris buffer and vortex mixing, might not enough to lyse all the amoebocytes since the cells, after lysis, were clamped together and might engulf

some cells within them which would protect the unlysed cells osmotic pressure. Therefore, five beads were added to the lysis method with the hope that this would assist uniformity of the cell lysis and give a better volume yield. The volume yield of P-04 and P-05 were compared to increase by 7.8% as there were both from female *T. gigas*. In experiment P-11, the lysis method was changed to freeze and thaw technique due to the observation that bead-vortexing technique caused some amount of lysate engulfed within the cell debris mass which would reduce the volume yield of the lysate. By the later technique the volume yield (comparison of P-11 and P-08) was increased by 3.6%. In order to reduce cost of lysate preparation, P-10 was used 0.025 M EDTA solution without Tris buffer, replacing it with water for injection. The result, as expected, gave firm gelation at the level comparable with that of Tris buffer.

There were two lots of horseshoe crabs (P-08 and P-09) from *T. gigas* and *C. rotundicauda* which were kept in sea water for the second haemolymph collection. Both of the lots were found to give no gelation reaction after the second collection. This might due to the fact that second collection possessed only young amoebocytes which their lysate could not give reaction with endotoxin which was reported by Jorgensen and Smith (3).

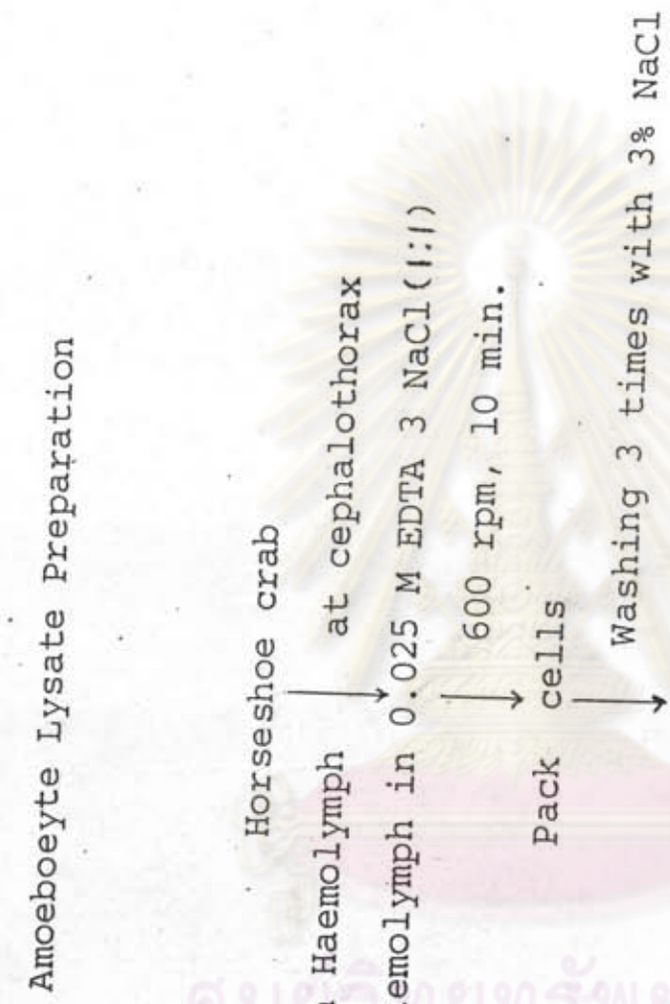
The eleven lots of preliminary experiments had given a standard protocol procedure for preparation of amoebocyte lysate as indicated in figure 10. With this procedure, five lots of female *T. gigas* and seven lots each of male *T. gigas* and seven lots of both

female and male *C. rotundicauda* were further carried out. The average volume of lysate per crab of female *T. gigas* was the highest value, and male *C. rotundicauda* was the lowest (table 3). The market price of the crabs from Samutphrakarn were seventy baht for female and thirteen baht for male of *T. gigas*, and ten baht for female and five baht for male of *C. rotundicauda*. Therefore, the maximum cost effective per crab was that of male *T. gigas* (2.44 baht per mL).

The osmolarity test of anti-clotting solution and the mixture was performed in order to confirm isotonicity of this solution with haemolymph. The result of osmolarity was indicated that anti-clotting solution was approximately isotonic with haemolymph.

The protein content of the lysate was statistically independent of both species and sex. The sensitivity of both species which gave firm gelation, was found to be the same at 0.1 ng/mL level of endotoxin and it was statistically no difference. At 0.05 ng/mL of endotoxin there was a high degree of variation in the gelation reaction. The uniformity of the reaction was found to be at 0.1 ng/mL as a minimum uniform detectable limit. The sensitivity of the lysate was sufficient enough for the use of the lysate in the detection of endotoxin at the level equal to that of the official limit of endotoxin presented in parenteral products (81, 82), and this sensitivity was comparable to that of commercial LAL which had been widely used (17, 83, 84).

Figure 10. Protocol procedure for amoebocyte lysate preparation.



We used magnesium ions for enhancement of the gelation reaction as the magnesium ion is a macro-component of synthetic sea water and gave the most reactivity when it was compared with other macro-components such as calcium, potassium, and bromide ions (76). The endotoxin levels detected with the addition of calcium ions did not correlated with rabbit pyrogen test whereas that of magnesium ions correlated with rabbit pyrogen test (85). The result of this study was that 50 mM of magnesium chloride concentration was optimal for using incorporation with the assay and the finding was in agreement with the report of Tsuji and Steindler (76). The 100 mM and 50 mM concentration was not statistically different in this study whereas the different was reported in Tsuji and Steindler's work. They used the system in which gave higher sensitive (end-point at 50 pg/mL) which was enhanced the different at very low level of endotoxin.

The incubation period of gelation reaction from both species could not be reduced shorter than 60 minutes period as stated by many investigators (3, 18) for tube method. The micro-test method which made used of defibrinated sheep red cells gave an unsatisfactory result even at 30 minutes period. The questionable results were obtained from the test gave a doubt whether this test was practical whereas the test showed to be clearly demonstrated by Hassaini and Hassanali (46). The incorporation of red cells was very difficult to control since the pressure applied to release red cells into the drops of the lysate mixture by micropipette, could not be uniformly controlled.

The specificity of the lysate from both species was performed with autoclave-killed organisms. The killing method was done for various organisms (table 9) to mimick the conditions encountered in usual sterilization process of pharmaceutical industries, and to obtain endotoxin as it might be occur in normal practice. The eight organisms (table 9) used in the study were represented the normal contaminants either from environment or human. From the result (table 21 and 22) gram negative bacteria, *E. coli*, *S. typhi*, *K. pneumoniae*, and *S. marcescens* gave firm gelation at the level of 10^5 or 10^4 cells/mL which were complied with the work of Devleeschouwer et al (77). The ten-fold difference of these bacteria might be due to the nature of endotoxin derived from these organisms (77, 86) and inaccuracy of the colony counting method (9, 13, 77). These variations were also reported in the literature (86, 87, 88). *S. aureus* which was gram positive bacteria should not give gelation reaction, we found it occurred at 10^6 cells/mL. The same observations were made by Wildfuer et al (78). This occurrence was explained by Devleeschouwer et al (77) that peptidoglycan of gram positive bacteria of high concentration could give reaction with the lysate, but the substance was less potent than control endotoxin used. In addition to the above reaction the contamination during organisms preparation might be taken into account. *Streptococcus*, which was also gram positive bacteria, gave very weak reaction with the lysate. The very weak reaction was again due to contamination, and insufficient destruction of the cell during autoclaving or a quantity of organisms too low to elicit the response.

This weak reaction should not be taken into account as the observation of sensitivity test that the weak gelation at 0.05 ng/mL was irregular and statistically difference from 0.1 ng/mL. The fungus, *C. albicans* possessed D-glucan component in the cell wall (89) which gave gelation reaction at 10^6 cells/mL due to the fact that lysate could give reaction with D-glucan as observed by Obayashi et al (19). This property of the lysate has given rise to a method used for detection of fungal polysaccharide (90).

The unknown parenteral products were obtained from one institution or purchased from drug stores. These products were grouped and coded from S-01 to S-25. Each tests were performed by using three samples and control as indicated in table 11, and the test was done three times as the result showed in table 23. The unknown samples, S-03 and S-06 which were 1,000 mL and 10 mL water of injection respectively, were observed to give weak reaction of gelation which indicated that endotoxin was in the level below 0.1 ng/mL. These products would pass rabbit pyrogen test, due to the fact that sensitivity of rabbit for endotoxin was about 0.1 ng/mL (16). The sub-febrile level of endotoxin in the test products could,if it occurred repeatedly, induce the animal to develop low level of tolerance and hence increased their pyrogenic level in further use of the animals. The unknown S-14 and S-21 were 5% dextrose in half strength saline and 5% dextrose in normal saline respectively. Both were in a package volume of 500 mL parenteral bottles. The observation (table 23) were found that S-14 and S-21 gave strong positive (grade

4) gelation reaction, indicated that S-14 and S-21 contained at least 0.1 ng/mL of endotoxin. The same observation was found with the commercial LAL. Unfortunately, the same lot of both unknown samples could not be obtained for quantitative end-point determination. Therefore, the exact amount of endotoxin presented in the samples could not be determined. Both unknown samples, S-14 and S-21, were presumed to pass pharmacopial rabbit pyrogen test as they were ready for consumption. The same observation were reported (17) that the product which pass rabbit pyrogen test might not pass LAL test. The proper maintenance of the rabbits in accordance with pharmacopial standard could be one of the factors responsible for the rising of pyrogenic threshold level. The group of unknown samples, S-23 to S-25, were paracetamol with lidocaine HCl injection locally produced in Thailand and packed in 2 mL ampoule. The detection of endotoxin using amoebocyte lysate test could not perform due to the fact that the inhibition test, which comprised of 0.01 mL 1ng/mL endotoxin and 0.1 mL of the unknown sample, gave no gelation reaction indicated a presence of inhibitors in unknown samples of this formula.

The lysate was kept in two forms namely, lyophilized and liquid forms. The commercial products of LAL were also kept in lyophilized form with instructions to store at 4°C. This storage conditions gave a shelf life of one year after the manufacturing date. Our lyophilized form was unsuccessfully tested for the activity, even immediately after the lyophilization process. The activity of the lysate was confirmed prior to the lyophilization, after which the lyophilized product possessed no activity even at the level of 100

ng/mL endotoxin. The previous literatures (7, 8) stated no difficulty in the performance of lyophilization process. Both CAL and GAL might contain a substance that destroyed enzymatic components of the lysate which activated during physical changes (liquid to solid, sublimations). In the liquid form at -20°C, the lysate could maintain activity only for 60 days after their preparation date (table 24). Even the lysate which was lysed with 5 mM Tris succinate buffer pH 7.4 could show activity only for 60 days. The gelation reaction was a enzymatic reaction (2, 17) which might be destroyed during storage conditions. The lysate prepared in this study was a crude lysate preparation which might possibly contain an inhibitor reacting on the active enzyme components responsible for gelation.

Based on this study, the standard protocol procedure for the amoebocyte lysate preparation was obtained to consist of 0.025 M EDTA in water for injection as a anti-clotting agent, 3% NaCl as an isotonic solution using in cell washing, and freeze & thaw technique for cell lysis (figure 10). The sensitivity of prepared lysate from *T. gigas* and *C. rotundicauda* were comparable with that of commercial available LAL. The specificity of the lysate to the autoclave-killed organisms was similar to that of LAL. The optimal incubation period of the lysate was at 60 minutes for tube method, and 30 minutes for that of micro test method. The micro test method was not practical due to that their visual end-point detection was irregular. The optimal magensium ion was found to be at 50 mM concentration. The endotoxin determination of unknown parenteral products was found to give weak

reactions (S-03, S-06), strong reactions (S-14, S-21), and non-specific inhibition reactions (S-23, S-25). The lysate prepared in the study could not be kept as long as that of commercial preparation. The lyophilized form of lysate lost all activity. The liquid form remained active for 60 days after preparation when stored at -20°C.