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PRODUCTION OF PHOSPHATIDYLINOSITOL PHOSPHOLIPASE C BY *BACILLUS SPHAERICUS*

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ABSTRACT. *Bacillus sphaericus* strains have been studied for production of an extracellular phosphatidylinositol hydrolyzing phospholipase C (PI-PLC). The enzyme activity was maximal at the end of exponential phase of growth. Specific activities of PI-PLCs secreted into the culture broth were between 0.011 and 0.038 U/mg protein. PI-PLC in the culture supernatant of *Bacillus sphaericus* was partial purified by 2-propanol precipitation and Sephadex G-75 gel filtration.

KEY WORDS: Phosphatidyinositol phospholipase C, *Bacillus sphaericus*, enzyme purification, entomopathogenic toxins

INTRODUCTION

Bacillus sphaericus is a Gram-positive sporeforming aerobic bacterium, common to soil and aquatic environments, that produces a binary toxin active against the larvae of *Culex* and *Anopheles* mosquitoes (Wirth et al., 2000; Smith et al., 2004). The binary toxin is composed of two polypeptides: BinA (41.9 kDa) and BinB (51.4 kDa), which are expressed in equimolar amounts during the early stages of sporulation. The binary toxin forms microcrystalline inclusions inside the mother cell that, once ingested, are solubilized in the alkaline pH of the larval gut and activated by larval proteases (Smith et al., 2004). The binary toxin is specifically bound to a unique receptor at the surface of the midgut epithelium of *Culex* and *Anopheles* larvae (Berry et al., 1993; Priest et al., 1997). The binary-toxin receptor has been identified as a 60 kDa glycosyl-phosphatidylinositol-anchored α-glucosidase present in the brush-border membranes of susceptible *C.pipiens* larvae (Poncet et al., 1999; Darboux et al., 2002).

In this article, we describe phosphatidylinositol-specific phospholipase C production from *Bacillus sphaericus* strains and discussed its correlation with pathogenicity.

MATERIAL AND METHODS

Bacteria and growth conditions. Nine strains *Bacillus sphaericus* were used. The strains were cultured in nutrient agar (National Center of Parasitic and Infectious Diseases, Sofia). For enzyme production each strain was grown in the liquid media of Zwaal et al. (1971), Gerasimene et al. (1981) and Taguchi et al. (1980), respectively, at 33°C, on a rotary shaker (100 rev/min).

Enzyme assay. PI-PLC activity was demonstrated by TLC (Ikezawa et al., 1976) and the method of Takahashi et al. (1981) with L-α-phosphatidylinositol (from soy been, 99%, Sigma) as a substrate. One unit PI-PLC was defined as the amount that would hydrolyse 1 μmol substrate per min at pH 7.0 and 37°C.

Purification of phospholipase C. After cultivation each culture supernatant was separated from cells by centrifugation at 12 000 (20 min, 0-4°C). The supernatant was concentrated by 2-propanol precipitation. The concentrated material was loaded onto a column (950 mm x 21 mn, i.d.) of Sephadex G-75 and eluted with 0.05 M Tris-HCl buffer (ph 7.8) at a flow rate of 20 ml/h. Fractions of 5 ml were collected throughout.

Protein analysis. Protein concentration was determined using the method of Hartree (1972) or Bradford (1976) with bovine serum albumin as a standard.

RESULTS

Bacillus sphaericus strains have been reported to secrete PI-PLC in the culture medium (Kostadinova, 2002). PI-PLC positive strains were cultured in the liquid medium, respectively, for 10-12 h. The cells were removed by centrifugation and the culture supernatants were assayed for activity using the method of Takahashi et al. (1981) as described previously (Kostadinova, 1997). The results is shown in Figure 1.

The most suitable medium for phospholipase C production was found to be a liquid media of Taguchi et al. (1980). *Bacillus sphaericus* strains NoNo 7 and 8 showed the highest activity – 0.034 and 0.038 U/mg, respectively. The correlation between bacterial growth and enzyme secretion is shown in Figure 2.

Phospholipase C secretion in the culture medium of *B.sphaericus* strain No 8 began at early log phase of bacterial growth and maximum enzyme activity was detected after 12 h cultivation (0.38 Units per ml). Changes of pH were observed. In late logarithmic phase of growth pH of the medium decreased (pH 6.0) and after that was detected alkalization (pH 8.0 after 20 h incubation) which coincided with PLC inactivation. This pattern of enzyme release during growth is similar to that observed with *B.sphaericus* strain No 7.

The enzyme of *B.sphaericus* No 8, which showed highest activity was purified by 2-propanol precipitation and Sephadex G-75 gel filtration (Table 1).

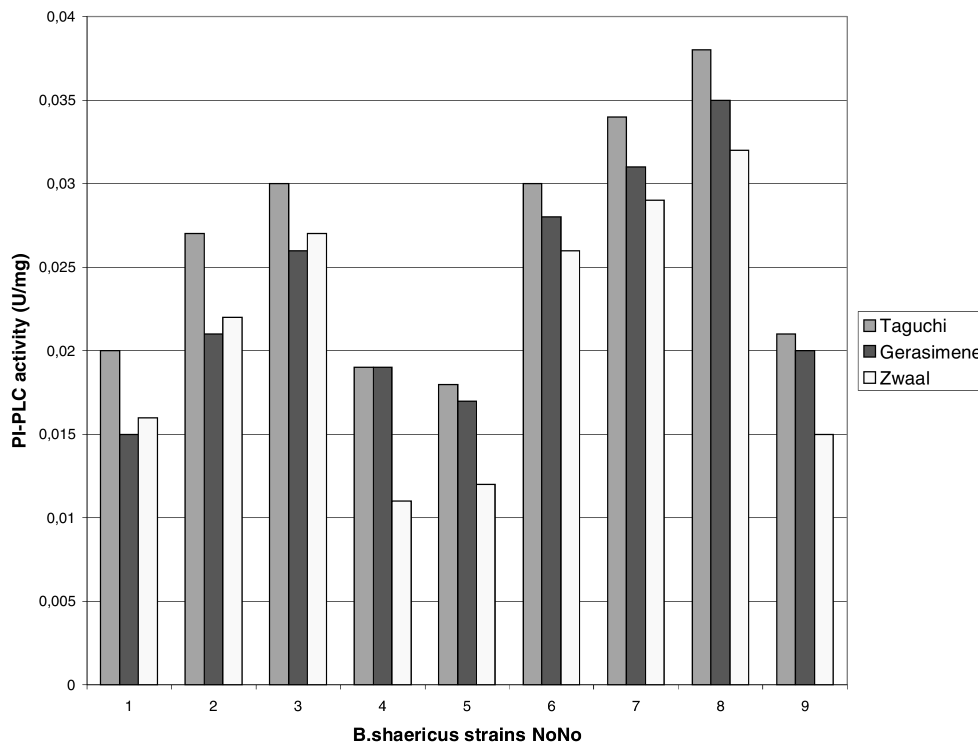

Fig. 1. Phosphatidylinositol-specific phospholipase C activity of strains *Bacillus sphaericus*. Protein contents were estimated by the method of Hartree (1972) with bovine serum albumin as a standard

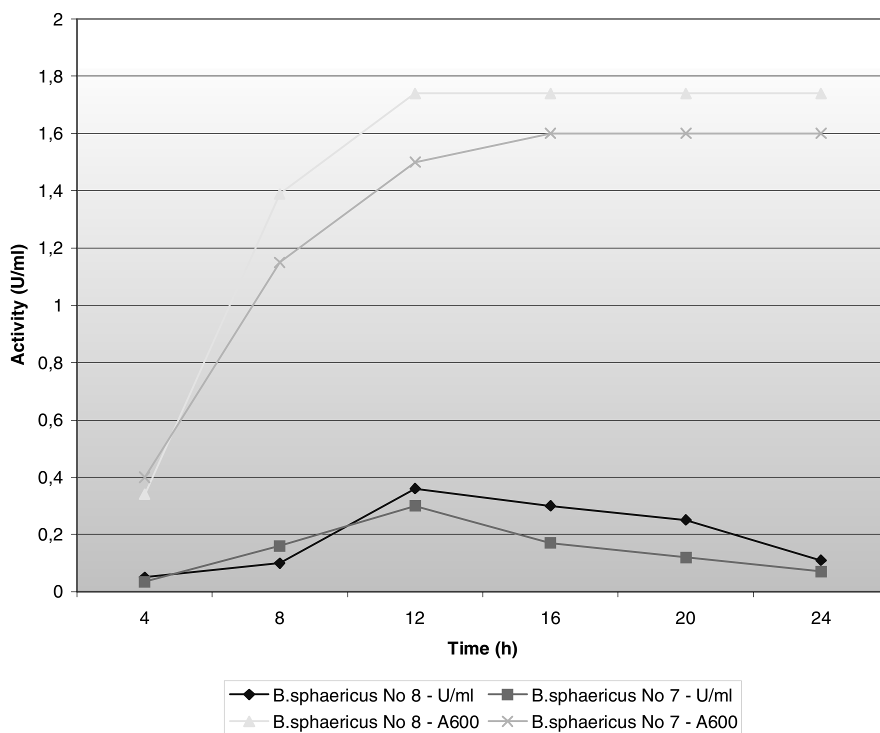

Fig. 2. Production of PI-PLC during the growth of *Bacillus sphaericus*. The activity was measured by the method of Takahashi et al. (1981) with L-α-phosphatidylinositol (Sigma) as a substrate

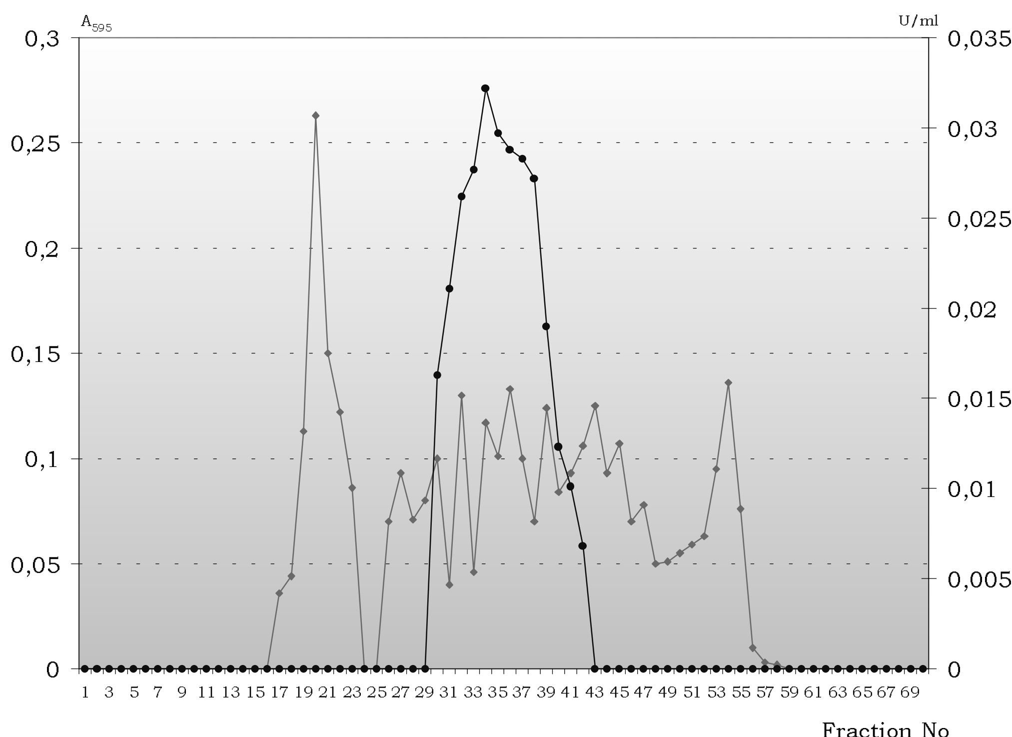

Fig. 3. Gel-filtration of *B.sphaericus* PI–PLC. The column was eluted with 0.05 M Tris-HCl buffer (pH 7.8) at a flow rate of 20 ml/h. (■) protein content; (♦) PI–PLC activity

In the supernatant of *B.sphaerucus* the proteins were reduced 9.9-fold and PI–PLC activity was increased 4.5-fold by 2-propanol precipitation.

The concentrated material was separated with Sephadex G-75 chtomatography. The column was equilibrated and eluted with Tris-HCl, pH 7.8 buffer (Figure 3). Phosphatidylinositol phospholipase C activity appeared in the minor protein fractions ranging from 150 and 210 ml. The specific activity increased 35 fold and the recovery was 56%.

The purification scheme resulted in a recovery of 25 % with a 160-fold increase in specific activity. The partial purified PI–PLC was free from phosphatidylcholine-specific phospholipase C and sphingomyelinase activities.

Table 1. Purification of PI–PLC from *Bacillus sphaericus* strain No 8

STEP	**PROTEIN (mg)**	**TOTAL ACTIVITY (U)**	**SPECIFIC ACTIVITY (U/mg)**	**PURIFICA-TION (fold)**	**RECOVERY (%)**
Supenatant	487	18.5	0.038	-	100
2-propanol precipitate	49	8.3	0.17	4.5	44.8
Sephadex G-75	0.76	4.7	6.1	160.5	25

* results are expressed as the mean of three experiments

DISCUSSION

The purpose of this work was to study phosphatidylinositol-hydrolysing phospholipase C produced by strains *Bacillus sphaericus*. In our previous work (Kostadinova, 2002) we found that *B.sphaericus* has phospholipase C activity. The highest PI–PLC activity was detected in the liquid medium of Taguchi et al. (1981) which was found to be a suitable for phospholipase C production from *B.cereus* and *B.thuringiensis* (Kamberov, Ivanov, 1990). The enzyme secretion was maximal at the end of exponential phase of growth. A similar pattern of production has been reported for phospholipase C of *B.cereus* and *B.thuringiensis* (Myrnes, Little, 1980; Kamberov, 1990).

PI-PLC from *Bacillus sphaericus* strain No 8 was partially purified. The initial activity of secreted phospholipase C was comparable with these reported for *B.thuringiensis/B.cereus* producers. By two steps procedure (a combination of 2-propanol precipitation and Sephadex G-75 chromatography) *Bacillus sphaericus* phospholipase C was purified 160 fold with 25% recovery. The purification procedure was effective in separating PI-PLC and other phospholipase C activities.

Phospholipases C have been found in a broad spectrum of bacteria, simple eukaryotes, plants and animals (Katan, 1998). Phospholipases play an important role in cellular signaling processes via the generation of second messengers such as diacylglycerols, arachidonate and inositol triphosphate.

Bacillus species produce large amounts of degradative enzymes at the end of exponential growth phase in response to unfavorable conditions of growth (Lereclus et al., 1996). *Bacillus thuringiensis* is known for its entomopathogenic properties which are due to the production of two structurally distinct droups pathogenic toxins – the crystal (Cry) δ-endotoxins and the cytolytic (Cyt) δ-endotoxins (Wirth et al., 2001). *Bacillus thuringiensis* and *B.sphaericus* were found to be toxic against similar hosts. The synergy between their toxins was demonstrated (Poncet et al., 1997).

Bacillus cereus, which is closely related to *B.thuringiensis* but does not produce crystal proteins, is facultative insect pathogen. Although the pathogenic mechanisms are unclear, it is assumed that the ability of these bacteria to develop in insects is partly due to their high production of phospholipases. Production of enzymes at the onset of stationary phase has a biological significance. The production of PI-PLC might allow the bacteria to damage and invade host tissues and gain access to novel sources of nutrients.

REFERENCES

BERRY, C., HINDLEY, J., EHRHARDT, A.F., GROUNDS, T., DE SOUZA, I., DAVIDSON, EW. 1993. Genetic determinants of host ranges of *Bacillus sphaericus* mosquito larvicidal toxins. J. Bacteriol., 175, 510-518.

BRADFORD, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-day binding. Anal. Biochem., 72, 248-254.

DARBOUX, I., PAUCHET, Y., CASTELLA, C., SILVA-FILHA, M., NIELSEN-LEROUX, C., CHARLES, J., PAURON, D. 2002. Proc. Natl. Acad. Sci. USA, 99, 5830-58-35.

GERASIMENE, G., MAKARUNAITE, U., KULENE, V., GLEMJA, A. 1980. Biosynthesis of extracellular phospholipase C (lecithinase) from *Bacillus cereus* depending on the nutrient medium composition and pH. Appl. Biochem. Microbiol., (Russia) 14, 523-526.

HARTREE, F. 1972. A modification of the Lowty method that dives a linear photometric response. Anal. Biochem., 48, 422-427.

IKEZAWA, H., YAMANEGI, M., TAGUCHI, R., MIYASHITA, T., OHYABU, T. 1976. Studies on phosphatidylinositol phosphodiesterase (phospholipase C type) of *Bacillus cereus*. I. Purification, properties and phosphatase-releasing activity. Biochim. Biophys. Acta, 450, 154-164.

KAMBEROV, E. 1990. Production, purification and properties of phospholipases type C of *Bacillus cereus* and *Bacillus thuringiensis.* (Ph.D Tesis), Sofia.

KAMBEROV, E., IVANOV, A. 1990. Purification of phospholipase C from *Bacillus cereus* by affinity chromatography on 2-(4-aminophenyl sulphonyl) ethyl-cellulose. J. Chromatog., 525, 307-318.

KOSTADINOVA, S. 1997. Phospholipase C from *Pseudomonas fluorescens B*. Biotechnol. Biotechnol. Eq., 11, 4, 38-42.

KOSTADINOVA, S., 2002. Lypolytic activity of strains *Bacillus sphaericus*. Trav. Sci. Univ. Plovdiv, 38, 7, 76-82.

KATAN, M. 1998. Families of phosphoinositide-specific phospholipase C: structure and function. Biochim. Biophys. Acta, 1436, 5-17.

LERECLUS, D., AGAISSE, H., GOMINET, M., SALAMITOU, S., SANCHIS, V. 1996. Identification of a *Bacillus thuringiensis* gene that positively regulates transcription of the phosphatidylinositol-specific phospholipase C gene at the onset of the stationary phase. J. Bacteriol., 178, 2749-2756

MYRNES, B., LITTLE, C. 1980. A simple purification scheme yielding crystalli-nephospholipase C from *Bacillus cereus*. Acta Chem. Scand., B 34, 375-377.

PONCET, S., BERNARD, C., DERVYN, E., CAYLEY, J., KLIER, A., RAPOPORT, G. 1997. Improvement of *Bacillus sphaericus* toxicity against dipteran larvae by integration, via homologous recombination, of the Cry 11A toxin gene from *Bacillus thuringiensis subsp. israelensis*. Appl. Environ. Microbiol., 63, 4413-4420.

PRIEST, FG., EBDRUP, L., ZAHNER, V., CARTER, P.E. 1997. Distribution and characterization of mosquitocidal toxin genes in some strains of B*acillus sphaericus*. Appl. Environ., Microbiol., 63, 1195-1198.

SMITH, A., CAMARA-ARTIGAS, A., ALLEN, J. 2004. Crystallization oh the mosquito-larvicidal binary toxin produced by Bacillus sphaericus, Acta Cryst., D60, 952-953.

TAGUCHI, R., ASAHI, Y., IKEZAWA, H. 1980. Purification and properties of phosphatidylinositol phosphodiesterase (phospholipase C type) of *Bacillus cereus*. Arch. Biochem. Biophys., 190, 1, 1-7.

TAKAHASHI, T., SUGAHARA, T., OHSAKA, A. 1981. Phospholipase C from *Clostridium perfringens*. Methods Enzymol., 71, 710-725.

WIRTH, M., WALTON, W., FEDERICI, B. 2000. Cyt1A from *Bacillus thuringiensis* subsp. *israelensis* respores toxicity of *Bacillus sphaericus* against resistant *Culex quinquefasciatus* (Diptera: Culicudae), J. Med. Entomol., 37, 401-407.

WIRTH, M., DELECLUSE, A., WALTON, W. 2001. Cyt1Ab1 and Cyt2Ba1 from *Bacillus thuringiensis* subsp. *israelensis* synergize *Bacillus sphaericus* against *Aedes aegypti* and resistant *Culex quinquefasciatus* (Diptera: Culicudae), Appl. Environ. Microbiol., 67, 7, 3280-3284.

ZWAAL, R., ROELOFSEN, B., COMFURIUS, P., VAN DEENEN, L. 1971. Complete purification and properties of phospholipase C from Bacillus cereus. Biochim. Biophys. Acta, 233, 474-479.