

Plant asparaginase-based asparagine biosensor for leukemia

Kuldeep Kumar¹, Mandeep Kataria² & Neelam Verma²

¹Biosensor Research Laboratory, Department of Biotechnology, M. M. Modi College, Patiala, Punjab, India, and ²Department of Biotechnology, Punjabi University, Patiala, Punjab

Abstract

The present work aims at the development of plant asparaginase-based asparagine biosensor for leukemia. It is a novel diagnostic for monitoring asparagine levels in patients suffering from acute lymphoblastic leukemia (ALL). Various immobilization strategies have been applied to improve the stability of the asparaginase. The latest and updated information including some new techniques of immobilization related to L-asparaginase such as gelatin, agarose, agar, and calcium alginate methods are described in detail along with response time studies and comparative data. Furthermore these immobilization techniques have been applied for the detection of asparagine in normal and leukemia serum samples.

Keywords: L-asparaginase, leukemia, biosensor, immobilized

Introduction

The enzyme L-asparaginase is widely used as antitumoral agent for the treatment of acute lymphoblastic leukemia and lymphosarcoma (Schemer and Holcenberg 1981). Normal cell grows well in the absence of L-asparagine, whereas tumor cells need this essential amino acid for their growth because these cells lack the enzyme asparagine synthetase that synthesizes L-asparagine (Asselin 1999) and in the presence of L-asparaginase, tumor cells cannot survive due to unavailability of important growth factor (Muller and Boos 1998). Lang (1904) was the first person to detect asparaginase activity in beef tissues. Kidd (1953) reported antitumor properties of Guinea pig serum, which was later attributed to asparaginase activity (Broome 1961). L-asparaginase is the first enzyme with antitumor activity to be studied in human beings. Some diseases, such as melanoma, reticulosarcoma, and Hodgkin disease, can also be treated well (Stecher et al. 1999). Thus ALL and lymphosarcoma are effectively treated by L-asparaginase and has been clinically accepted as antitumor agent (Mashburn and Wriston 1964).

The enzyme L-asparaginase is produced by a variety of microorganisms such as *Erwinia cartovora* (Maladkar et al. 1993), *Escherichia coli* (Derst et al. 1994), *Candida utilis*

(Kil et al. 1995) and *Thermus thermophilus* (Prista and Kyriakidis 2001). Among various plants species, production of L-asparaginase has been reported. *Withania somnifera* was identified as potential source of L-asparaginase due to high specific activity (Oza et al. 2009). The presence of an amidase in barley roots capable of hydrolyzing L-asparagine studied the distribution of L-asparaginase (Grover and Chibnall, 1927). L-asparaginase was also produced in large amount from root nodules of soybean (Streeter 1977). *Capsicum annum* and *Tamarindus indica* contain appreciable amount of L-asparaginase (Bano and Sivaramakrishnan 1980) and was also detected in young leaves of *Pisum sativum* (Siechiechowicz and Ireland 1989). L-asparaginase activity was also detected from two more plants *Lupin arboreus* and *L. angustifolius* (Borek et al. 1999).

Fabrication of asparaginase biosensor is a promising technology to detect L-asparagine. Garlic tissue electrode is used to determine L-asparagine where garlic tissue cells were employed for conversion of L-asparagine into ammonia and ammonium gas electrode was used as detector (Kim et al. 1995). A thermostable recombinant asparaginase from *Archaeoglobus fulgidus* was cloned and expressed in *E. coli* as a fusion protein and the immobilized enzyme was used with an ammonium selective electrode to develop a biosensor for L-asparaginase (Wang et al. 2002). A garlic (*Allium sativum* L.) peroxidase biosensor for hydrogen peroxidase monitoring, which was immobilized on chitosan matrix, was formed (El Ichi et al. 2008). *Petunia punctata*, *Alternanthera sessilis* and *Amoora chittagonga* extracts showed cytotoxicity screening against three pancreatic cancer lines—adenocarcinoma cell line Panc-1, Mia-Pacca-2, capan-1 using label free biosensor assay (George et al. 2010). Amperometric oxalate biosensor based on sorghum leaf oxalate oxidase, immobilized on carboxylated multiwalled carbon nanotubes, conducting polymers, and polyaniline composite film was constructed (Yadav et al. 2011). The current study presents the development of a novel, diagnostic and cost-effective plant L-asparaginase-based biosensor for leukemia blood samples. Color visualization approach has been optimized to monitor asparagine levels.

Materials and methods

The enzyme L-asparaginase that was used for the fabrication of biosensor to monitor asparagine levels in blood serum samples of normal healthy person and in patients suffering from acute lymphoblastic leukemia and lymphosarcoma was extracted from plant, *Withania somnifera* (L.) Dunal. The obtained crude extract (L-asparaginase) was coimmobilized with phenol red indicator that changes color from red to violet (red color was due to phenol red indicator). When the enzyme L-asparaginase come in contact with substrate (asparagine), deamination reaction takes place in which asparagine break down into aspartic acid and NH_4^+ ions which were responsible for change in color. Asparagine concentrations ranging from 10^{-10} – 10^{-1} M were studied by different techniques through change in color by visualization approach. In biosensor construction strategies, L-asparaginase was coimmobilized with phenol red indicator by gelatin method, agarose method, agar method, and calcium alginate beads formation method. Storage stability of immobilized plant L-asparaginase was studied.

Immobilization techniques

Gelatin method

Dissolve 1.0 g gelatin in 10 ml of water to prepare a 10% aqueous solution. Heat the solution gently to facilitate the dissolution process. Approximately 20 μl of enzyme (0.5 U) and 2 ml of hardening solution (20% Formaldehyde, 50% Ethanol, 30% Water) were added followed by 10 μl of phenol red indicator to the solution. Then pour it into a mold and allow it to freeze at -20°C for 4 hours to facilitate the gel formation. When the gel is set, warm the gel to room temperature. Then cut the gel into small blocks of approximately 3 mm per side (Alteriis et al. 1985). Put these blocks into varying concentration of L-asparagine and the response time is noted for change in color of blocks from partly orange to dark purple.

Agarose method

Agarose solution (1.5%) is prepared in 25 mM Tris- acetate buffer (pH 7.2) containing 2 mM CaCl_2 by heating for 10vminutes. Then 20 μl enzyme (0.5 U)/10 ml above solution is added followed by 10 μl phenol red indicator.

Solution is poured into petriplate and allowed to solidify. The gel is then cut into small pieces of 1.0×1.0 cm (Prakash et al. 2007). The gel pieces are put into varying concentration of L-asparagine (10^{-10} – 10^{-1} M) and the response time is noted for change in color of small pieces from partly orange to dark purple.

Agar method

A solution of 4% agar is prepared. Boil it and allow it to cool at 45 – 50°C . 20 μl enzyme (0.5 U) and 10 μl phenol red indicator is added to the solution. Mix it thoroughly and pour it into petriplate and allow it to solidify. The gel is then cut into square cakes of 1.0×1.0 cm with the help of knife or spatula (Mahajan et al. 2010). Then the cakes are put into varying concentration of L-asparagine and the response time is noted for change in color of cakes from partly orange to dark purple. Detection limit of L-asparagine achieved was 10^{-10} – 10^{-1} M.

Calcium alginate beads

Slurry of 3% sodium alginate with 20 μl of the enzyme solution (0.5 U) is formed and 10 μl of phenol red indicator are added to this slurry. This solution is then poured drop wise through a glass syringe into a beaker containing 0.075 M chilled CaCl_2 with gentle stirring on a magnetic stirrer. Orange color beads (partly orange color of the beads was due to phenol red indicator) are made with the help of 2.5 ml syringe without needle (Johnsen and Flink 1986). Harden the beads by placing it for half an hour at room temperature. 5 to 6 beads are put into varying concentrations of L-asparagine (10^{-10} – 10^{-1} M) solutions. The response time for change in color of beads from partly orange to bright purple is then noted.

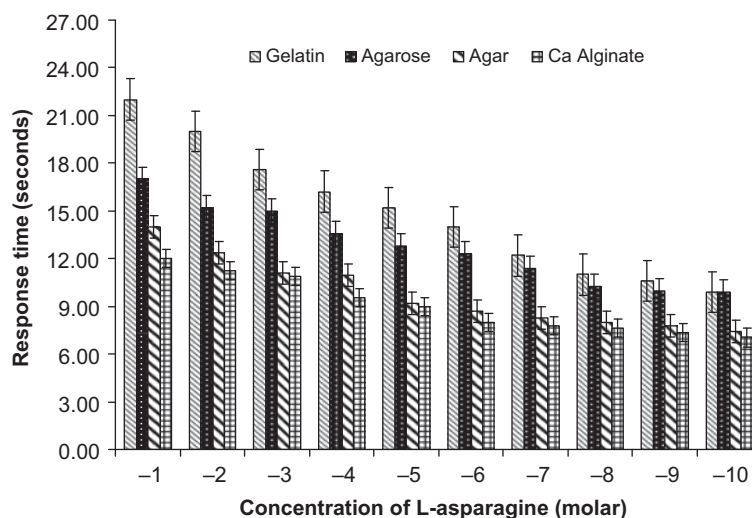
For monitoring asparagine levels in normal and leukemia blood serum samples, few beads of calcium alginate are put into both the samples. Response time for color change of beads is noted and compared with change in response time with in concentration levels.

Storage stability

To know the storage stability of biocomponent, gelatin gel blocks, agarose pieces, agar cakes, and calcium alginate beads blocks were kept in refrigerator. The activities of immobilized biocomponents were checked.



Figure 1. (A) Comparison of color of gelatin blocks (Before and after the reaction) (B) Leukemic Blood Sample.



Graph 1. Performance of biosensor with L-asparagine standards (10^{-1} – 10^{-10} M) using different matrix.

Results and discussion

Gelatin method

For gelatin method, visual color change was observed. Figure 1 shows comparison of color of gel blocks before and after the reaction. Detection limit of asparagine achieved was 10^{-10} – 10^{-1} M. For concentration level of 10^{-1} M L-asparagine, response time detected was 22 seconds and for the concentration level of 10^{-10} – 10^{-2} M L-asparagine, response time detected was in the range of 10–20 seconds (Graph 1). Response time decreased with decrease in concentration of asparagine indicating more of NH_4^+ ion produced after hydrolysis.

Therefore, these gel blocks have been used for subsequent experiments of optimization of the response time and monitoring asparagine levels in normal and leukemia serum samples. Response time for change in color of blocks was 16 seconds for normal blood sample. The L-asparagine concentration level was 10^{-4} M. For leukemic blood samples, response time for change in color of blocks was 19 ± 0.5 seconds corresponding to 10^{-2} M L-asparagine.

Agarose method

For agarose method, visual color change was observed. Figure 2 shows comparison of color of the gel blocks before and after the reaction. Detection limit of L-asparagine

achieved was 10^{-10} – 10^{-1} M. For concentration level of 10^{-1} M L-asparagine response time detected was 17 seconds and for the concentration level of 10^{-10} – 10^{-2} M L-asparagine, response time detected was in the range of 10–15 seconds (Graph 1). The response time for change in color was found to be directly proportional to the asparagine concentration levels.

Response time for change in color of gel pieces was 13 seconds for normal blood sample. The L-asparagine concentration level was 10^{-4} M. For leukemic blood samples, response time for change in color of gel pieces was 15 seconds corresponding to 10^{-2} M L-asparagine.

Agar method

Detection limit of L-asparagine achieved was 10^{-10} – 10^{-1} M. For concentration level of 10^{-1} M asparagine, response time detected was 14 seconds and for the concentration level of 10^{-10} – 10^{-2} M L-asparagine response time detected was in the range of 7–12 seconds (Graph 1). With decrease in concentration of asparagine, the response time for color change increased. Visual color change was observed in Figure 3.

Response time for change in color of gel blocks was 11 seconds for normal blood sample. The L-asparagine concentration level was 10^{-4} M. For leukemic blood samples, response time for change in color was 12 seconds corresponding to 10^{-2} M L-asparagine.

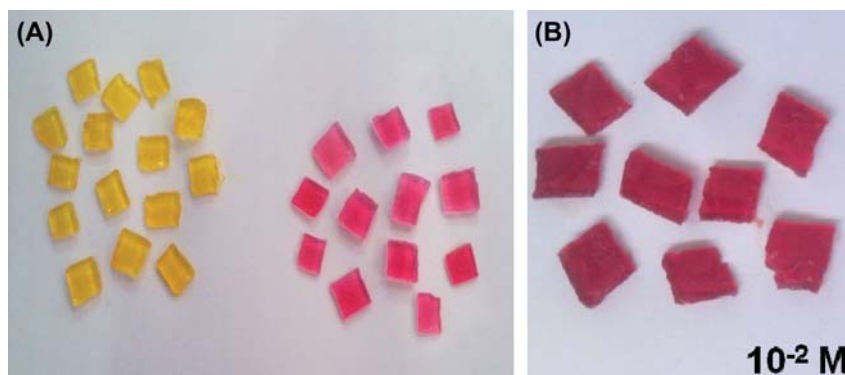


Figure 2. (A) Comparison of color of agarose gel pieces (Before and after the reaction) (B) Leukemic Blood Sample.

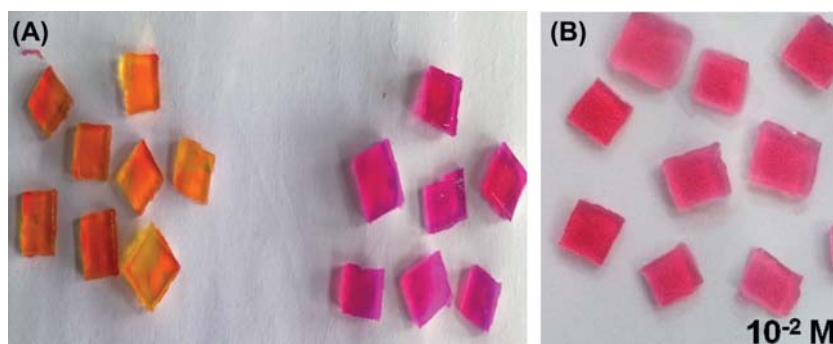


Figure 3. (A) Comparison of color of agar blocks (Before and after the reaction) (B) Leukemic Blood Sample.

Calcium alginate beads

For calcium alginate beads, visual color change was observed. Figure 4 shows comparison of the color of beads before and after the reaction. Detection limit of L-asparagine achieved was 10^{-10} – 10^{-1} M. For concentration level of 10^{-1} M L-asparagine, response time detected was 12 seconds and for the concentration level of 10^{-10} – 10^{-2} M L-asparagine, response time detected was in the range of 7–11 seconds (Graph 1). This is directly related to decrease in NH_4^+ ions produced after the reaction due to increasingly lesser concentration of the reactant.

Response time for change in color of beads was 9.3 seconds for normal blood sample. The L-asparagine concentration level was 10^{-4} M. For leukemic blood samples, response time for change in color of beads was 11 seconds corresponding to 10^{-2} M L-asparagine.

Storage stability of the biocomponent

The biocomponent was found to be active. Biocomponent in immobilized gelatin gel blocks, agarose pieces, agar cakes, and calcium alginate beads was found to be stable for long time that is more than 4 days, 12 days, 4 days and 2 months respectively. Thus immobilization in calcium alginate beads was considered to be the most reliable and effective method.

Conclusion

The developed biosensor using various immobilization techniques was able to detect asparagine levels from

10^{-10} to 10^{-1} M and further it was used for the detection of asparagine levels in normal and leukemia blood serum samples. Out of all the four immobilization techniques, calcium alginate bead method was found to be best among them. As the observable response time was 12 seconds, which is least in all the four techniques. In comparison with *E. coli* K-12 asparaginase-based asparagine biosensor developed by Verma et al. (2007) could detect asparagine levels of 10^{-9} M while some earlier efforts could detect levels up to 10^{-5} M only (Borek et al. 1999). Plant biosensor is found to be better. This is because the biosensor formed with *E. coli* K-12 gives more response time as compared to plant biosensor. Thus, the developed plant-based biosensor is novel, diagnostic, very rapid, easy to use, inexpensive, portable, and capable of nanolevel asparagine detection.

Acknowledgement

The authors wish to thank Modi Education Society and Dr. Satish K. Bhardwaj, Principal, M.M. Modi College, Patiala for their encouragement.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

The present work was supported by University Grant Commission [F.No.8-3(223)/2011 (MRP/NRCB)].



Figure 4. (A) Comparison of color of Calcium alginate beads (Before and after the reaction) (B) Leukemic Blood Sample.

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