Isolation Of Cassava Linamarin As A Minimal Immuno-Suppressive Drug Against Cancer

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Abstract - Cancer is a disease caused by some physical, chemical or biological factors that leads to unregulated proliferation of cells resulting in the formation of malignant lesions. Although there are a number of treatment methods available against cancer, majority of them have side-effects. The use of natural remedies for developing a chemo-therapeutic drug that is minimally immunosuppressive but selectively kills the cancerous cells is the major objective of the research. Cassava (Manihot esculenta Crantz) has been proven to be a natural remedy against cancer due to the cyanogenic compound linamarin present in it. The isolation of cassava linamarin by acid hydrolysis followed by adsorption on activated carbon can produce highly purified end-product that can be used as an anti-cancer agent. The acid hydrolysis was carried out using 0.1M H₂SO₄ and the crude cassava extract (CCE) obtained was analyzed for functional groups using FTIR spectroscopy. The hydrolyzing solution was prepared using 0.1M acetate buffer and the enzyme obtained was purified using membrane filtration (0.45µm). For quantification of the linamarin present in CCE, UV-Vis spectroscopy was conducted to obtain the calibration curve (absorbance versus concentration curve) from which the concentration of linamarin present in the sample measured in hydrogen cyanide equivalent terms was found to be 0.97µg for 0.1ml of CCE used that was hydrolyzed using 11ml of hydrolyzing solution containing linamarase. The UV-Vis spectroscopic studies revealed that the optimum time required for the purification of linamarin using activated carbon adsorption was 35 minutes. Thus, an approach for the isolation and purification of linamarin by combining acid hydrolysis and activated carbon adsorption technique was proposed.

Keywords – cassava; anti-cancerous drug; linamarin, activated carbon adsorption

I. INTRODUCTION

Cassava (Manihot esculenta Crantz), a carbohydrate rich source is the third most important staple food crop in the developing countries after rice and maize [1]. Cyanogenic compounds in cassava, mainly linamarin and lotaustralin are synthesized and distributed primarily for defensive purpose against predators across the whole plant [2]. Linamarin being predominant among them is present in greater concentration in root peels than in edible tuber portions [3,4]. The concentration of cyanogenic compounds varies depending on the cultivated variety, environmental conditions and growth factors [5,6].

Cancer, a disease where normal cells undergo vigorous proliferation to generate metastatic malignant tumor cells that give rise to secondary tumors may be due to various chemical, physical, biological or environmental factors leading to the disturbance in normal functioning of human body [7,8,9,10]. Even though various chemotherapeutic drugs are available for the treatment of cancer, the immune response of the patient is suppressed thus causing more damage to the patient than curing it [10,11]. A chemotherapeutic drug having minimal immunosuppressive effects has been a major focus area for the researchers. Crude cassava extract has been used for the treatment and control of cancer in ancient times [12]. Along with cyanogenic compounds the cassava tissues also contain the hydrolyzing enzyme linamarase that hydrolyzes the substrate only during cell rupture [13]. The hydrolysis results in the release of a molecule of sugar and ketone, namely glucose and acetone and a molecule of hydrogen cyanide [14].

In normal somatic cells, the cyanide released during the hydrolysis of linamarin by beta-glucosidase immediately get detoxified by the mitochondrial rhodanase enzyme that convert it into less toxic form which is expelled from the body through sweat, tear, urine, serum etc. [10,11,15,16]. But in cancer cells, due to their high nutrient requirement will be rich in hydrolyzing enzyme but devoid of detoxifying enzyme, selective accumulation of cyanogenic compound...
occurs finally leading to cyanide poisoning of cancer cells [10,11].

Currently there are few studies that deal with isolation of linamarin from cassava [17]. For the quantitative determination of linamarin in the cassava extract, enzymatic hydrolysis using linamarase followed by spectroscopic analysis can reveal the linamarin concentration in terms of hydrogen cyanide equivalent released. Rather than chemical purification of the compound, the use of membrane filtration and activated carbon adsorption can be employed for the removal of impurities present in the sample [18,19].

II. MATERIALS AND METHODS
A. Chemicals required

0.1M sulphuric acid, sodium carbonate, picric acid, 0.1M phosphate buffered saline solution (PBS) (pH 6.0), and 0.1M acetate buffered solution (pH 5.5), 0.2M phosphate buffered solution (pH 7.2), ammonium sulphate (60% w/v) commercial granular activated carbon and distilled water. The chemicals were used as received without further purification.

B. Sample preparation

Fresh cassava cortex tissues were pooled, washed and diced to small cubes. The air dried samples were powdered and blended with 0.1M sulphuric acid. After preliminary separation of macromolecules using a filter cloth, the filtrate was homogenized again and filtered with filter paper. The solution was centrifuged at maximum speed till the clear supernatant was obtained that was stored at 4°C.

Alkaline sodium picrate solution was prepared by taking 250mg Na2CO3 and 50mg picric acid and dissolving it in 10ml of distilled water. Whatman filter paper was cut into size and dipped into the solution which immediately turned from white to yellow. After 30 minutes, the filter papers were removed and dried, later stored in a dark medium until use.

For preparation of hydrolyzing solution, powdered cassava root peels were homogenized using 0.1M acetate buffer (pH 5.5) until it became a paste. The mixture was filtered and centrifuged to collect the supernatant. Ammonium sulphate precipitation using 60% (w/v) ammonium sulphate was carried out at low temperature to precipitate out proteins [20]. The mixture was centrifuged at 3000rpm for 1hr to collect the precipitate that was then dissolved in 0.1M phosphate buffered saline solution (pH 6). The enzyme was purified using membrane filtration (0.45μm) and stored at 4°C.

C. Characterization of cassava linamarin

The crude cassava extract was analyzed using Perkin Elmer Fourier Transform Infra-Red (FTIR) (Spectrum Version 10.4.00) analyzer. UV-Vis spectroscopic analysis was conducted by adding varying concentration (3-13ml) of hydrolyzing solution to test tubes containing 0.1ml of crude cassava extract (CCE) in 0.5ml of distilled water with 10ml of 0.2M phosphate buffer (pH 7.2). A yellow picric paper was suspended above each sample and the test tubes were immediately stoppered. After overnight incubation at 30°C, the papers were removed and immersed in minimal amount of distilled water. After 30 minutes, elutes were collected and the absorbance was read at 200nm against a picric paper blank using UV-Vis spectroscopic analyzer.

D. Purification using activated carbon

8g of crude cassava extract (CCE) was introduced into 8g of activated carbon and the mixture was shaken (200 rpm) at room temperature for varying time interval (0-100 minutes) and filtered. After each contact time and filtration, 4 ml filtrate added to 11 ml hydrolyzing enzyme solution and analyzed.

III. RESULTS AND DISCUSSIONS
A. FT-IR characterization of the crude cassava extract

For the inactivation of the endogenous enzyme, linamarase the acid extraction is carried instead of alkaline extraction since the acid extracts are more stable [5]. In addition, the concentration of linamarin in the extract of the root cortex was higher than that of the root parenchyma. The crude cassava extract was prepared in acidic condition by inactivating the hydrolyzing enzyme followed by filtration and centrifugation for the removal of macromolecules. The FTIR spectrum of CCE was obtained as shown in fig.1. From the FT-IR spectrum a broad absorbance at 3339.09cm⁻¹, a small peak at 1634.91cm⁻¹ and a weak peak at 2170cm⁻¹ were observed. The broad peak within indicates the presence of variable alcohol groups. The small peak at 1634.9cm⁻¹ indicates the presence of –CHO group. The weak peak approximately at 2170cm⁻¹ reveals the presence of -C≡N bond.

B. Determination of optimum amount of hydrolyzing solution

For the hydrolysis of linamarin from the crude cassava extract, a 0.45μm membrane was used for the purification of the enzyme from solvent. The impurities were removed from the hydrolyzing solution while the enzyme gets concentrated during
the membrane separation process. The picrate paper color after incubation turned from yellow to brown. The presence of linamarin in the solution was confirmed when the color change occurred during the addition of portions of the hydrolyzing solution to the CCE.

To study the effect of enzyme concentration on the reaction rate, the concentration of the substrate, i.e., linamarin was made to be present in excess. The concentration of the enzyme solution was varied from 3 to 13ml to find the optimum concentration. The amount of linamarin present in the sample was measured in terms of the activity of linamarase that hydrolyses it to release HCN or glucose, or in other terms as hydrogen cyanide equivalent concentration by the spectrophotometric technique. The fig.2 confirms the activity of linamarase as an enzyme that breaks down linamarin by hydrolyzing it to release HCN and glucose. The reaction is proportional to the amount of the hydrolyzing solution used. The highest activity was noted when 11 ml of enzyme solution was used, where it started leveling off at 0.97µg. This is regarded as the HCN equivalent concentration released during the reaction.

Further increase in the amount of enzyme dose not increases the reaction rate. Since linamarin was used in excess, 11 ml of the enzyme solution was determined to be the optimum hydrolyzing amount.

**C. Adsorption point of linamarin**

The purification of linamarin using excess amount of activated carbon was conducted to determine the adsorption point. The fig.3 shows the variation in concentration of linamarin with time. During the purification process, the organic impurities present in the crude cassava extract tend to have more affinity for activated carbon than linamarin. Thus at the initial stages, the impurities gets adsorbed and a point is reached where the linamarin concentration is the highest. This point termed as adsorption point is a particular time at which all the impurities get adsorbed and linamarin in the sample gets purified. Beyond the adsorption point, the decrease in linamarin concentration reveals the adsorption of linamarin on activated carbon. For 8g of CCE used, the linamarin gets purified at approximately 35 minutes.
IV. CONCLUSION

The use of crude cassava extract for cancer treatment in folk medicines indicated the potential of the cyanogenic compounds in the extract as anti-cancer agents. The hydrogen cyanide released during the hydrolysis of linamarin can preferentially kill the cancer cells, thus a possible chemotherapeutic drug having minimal immunosuppressive effects can be developed. The research aims at an easy and efficient method for isolation and purification of cassava linamarin. The method involves acid hydrolysis of the cassava root peel and purification using activated carbon adsorption. The enzyme, linamarase used for hydrolysis of linamarinhas been purified using membrane filtration technique and optimized to be 11ml for CCE sample taken. The characterization of linamarin using FTIR indicates the presence of different functional groups that are present in linamarin. Optimum contact time with activated carbon during linamarin purification with the crude cassava extract (CCE) was found to be 35 minutes approximately. Because of the sensitive nature of linamarin in normal conditions, extreme handling care must be taken when processing it within the room temperature and must be stored at very low temperature. Clinical studies must be conducted to check the effectiveness of linamarin as an anti-cancer agent.

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References