ENZYMATIC EXTRACTION OF LOW METHOXYL PECTIN AS A POTENTIAL ANTI CANCER AGENT FROM GREEN CINCAU (*Premna Oblongifolia Merr.*)

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Abstract

Pectin is used in a number of foods as gelling agent, thickener, texturizer, emulsifier and stabilizer. It is also used in pharmaceutical, dental and cosmetic industries for its jellifying properties. Commercial pectin is divided into high and low methoxy pectin. Low methoxyl pectin (LMP) is reported posses anti cancer activity. One of our local resources, green cincau (Premna oblongifolia Merr.) is one of source of LMP. Due to its wide spectrum of functional properties and its potency as anti cancer compound, hence it is urge to develop a production process of LMP from green cincau. The current technology of pectin extraction is acidic hydrolysis. It has at leat two demerits: it does not allows pectin to be extracted fully with no damage to its structure and it does not meet the environmental safety. An enzyme-hydrolytic technology seems environmentally safe and more effective in terms of pectin yield. But nowadays the main demerits of the enzymatic extraction is the high price of commercial enzyme. In order to overcome all the demerits of pectin extraction, it is proposed to enzymatically extract the pectin of green cincau by using enzyme isolated from the local resources such as cellulase which can be isolated from hepatopancrease of snail and protease isolated from Calotropis gigantea.

Key Words: *enzyme, extraction, low methoxyl pectin, anti cancer, green cincau*

INTRODUCTION

Pectin is defined as a mixture of heteropolysaccharides. The polysaccharide structure is based on 1,4 linked α-D-galacturonic acid, interupted by L-rhamnose, L-arabinose and D-galactose. Pectin is food additive widely used in the food industry because its gelling, stabilizing, thickening and emulsifying properties. It is also used in pharmaceutical, dental and cosmetic industries for its jellifying properties (Baississe, et al., 2010).

Commercial pectin is currently classified according to the degree of esterification (DE). There are three classifications of pectin: HM (high ester); LMC (low ester conventional) and LMA (low ester amidated). High methoxyl pectin have usually a more than 50% share of esterified polygalacturonic acid units (DE°), while low methoxyl pectin have usually less than 50% share of esterified polygalacturonic acid units (DE°).

Many researchers stated that LMP posses anti cancer activity. Many recent studies are showing that administering pectin could reduce the risk of cancer, or even halt the progression of cancer. In the case of one study, administering pectin to cancer cells inhibited the growth of new cancer cells, and in a certain percentage of cases caused the cancer cells to start to die. If this can be developed further, pectin administration could be a good co-treatment for chemotherapy or radiation, possibly allowing for a reduction in the amount of chemotherapy or radiation required. This would be a positive advancement, due to the high toxicity to the body of both chemotherapy and radiation.

The bulk of the studies that address pectin and cancer center around its apparent ability to bond with a particular protein called galectin-3. Galectin-3 is a protein that has been recognized as a cancer-causing agent. What happens is the galectin-3 molecules start to bond together. Once they bond together, cancer cells start forming as a result of the clumped proteins. When pectin is administered, the pectin bonds to the individual galectin-3 molecules, inhibiting clumping. The galectin-3 then passes out of the body, with the pectin (Sundeen, 2009).

Raw materials which are source of pectin are pomace, sugar beet chips and citrus peels. One of our local resources that could be used as raw material for low methoxyl pectin is greeen cincau (Premna oblongifolia Merr.). The gel forming component of cincau extract and its fractions are mainly composed by low methoxyl pectin hydrocolloid (Nurdin, 2005). Plants cell walls such as cell walls of green cincau is consist of a series of layers, from outer to inner, respectively, are the

middle lamella, primary cell wall, secondary wall and plasma membrane. The highest concentration of pectin is seen in the middle lamella estimated to be in the order of 10-30% (Wang et al., 2002).

Pectin is generally produced by acid extraction of citrus peel followed by filtration and precipitation by alcohol as 2-propanol. Conventionally, extraction of pectin is performed at about 90°C for at least 1 h. Unfortunately, these conditions lead to protein degradation and are not good for either quantity or quality of pectin extracted (Rezzouk, 2008). Acid ectractionalso has at leat two demerits: it does not allows pectin to be extracted fully with no damage to its structure and it does not meet the environmental safety. An enzyme-hydrolytic technology seems environmentally safe and more effective in terms of pectin yield (Ptichkina, 2008). But nowadays though enzymatics extraction can result in high yield and selectivity (Huey, 2008; Troger, 2010) it has the main demerits of the enzymatic extraction which is the high price of commercial enzyme (Troger, 2010). In order to overcome all the demerits of pectin extraction, it is proposed to enzymatically extract the pectin of green cincau by using enzyme isolated from the local resources such as cellulase which can be isolated from hepatopancrease of snail and protease isolated from Calotropis gigantea.

This paper is intended to give a short description on green cincau, pectin, pectin mechanism in preventing cancer, pectin extraction and enzymatics extraction of pectin.

Green Cincau (Premna oblongifolia Merr.)

Green cincau leaf (Premna oblongifolia Merr) is commonly extracted with water to prepare a fibre drink containing polysaccharide forming gel. The leaves of green cincau (Figure 1) have been shown to have antioxidant activities in in vitro and in vivo experiment and have minimum possibility of producing radical oxidation products which provide evidence of the safety aspect of this food.

Figure 1. Premna oblongifolia Merr.

The gel forming component of cincau extract and its fractions are mainly composed by low methoxyl pectin hydrocolloid. Low methoxyl pectin are physically bound in situ via metallic cations, especially divalent cations (Nurdin, 2005).

The extraction of pectin from green cincau leaves by using nitric acid conducted by Nurdin et.al. (2005) showed a high yield and pectin content. On the utilization of 0,2% nitric acid, the yield and the pectin content is up to 21% and 47%, respectively (Nurdin, 2005).

Pectin

Pectin is a major cell wall component with a variety of important biological functions in plants. It plays a role in the control of cell growth, in defense against invasions of microorganisms and in maintaining the physical and sensor properties of fresh fruits and their processing characteristics.

Plant cell walls consist of a series of layers, from outer to inner, respectively, are the middle lamella, primary cell wall, secondary cell wall (in some of the plants) and plasma membrane (Figure 2). Cell walls contain approximately 60% water and 40% polymers, of which pectins make up 20–35%. The highest concentration of pectin is seen in the middle lamella, with a gradual

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decrease in passing through the primary cell wall toward the plasma membrane. The concentration of pectin in the middle lamella is estimated to be in the order of 10–30% (Wang, 2002).

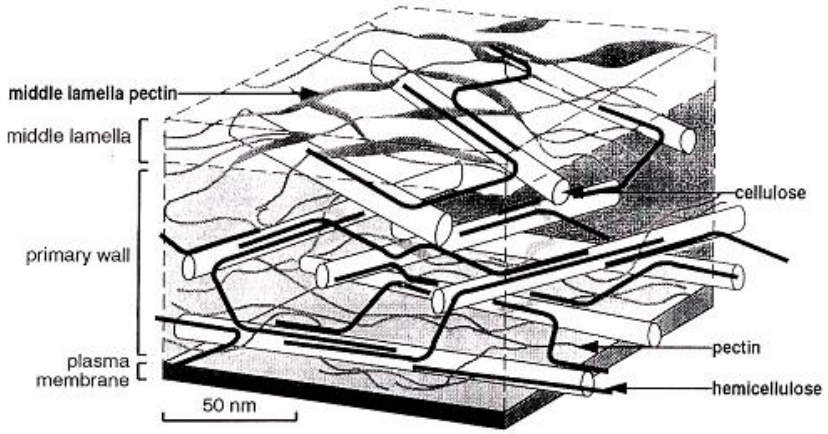

Figure 2. Simplified and Schematic Representation of the Architecture of the Cell Wall.

Pectin and cancer

Chemically, pectin is known as a long-chain polysaccharide, a string of molecules comprised primarily of sugar (Figure 3). Given its constitution, pectin is particularly attractive to molecules that bind with galactose and among these molecules is a class of carbohydrate-binding proteins called galectins.

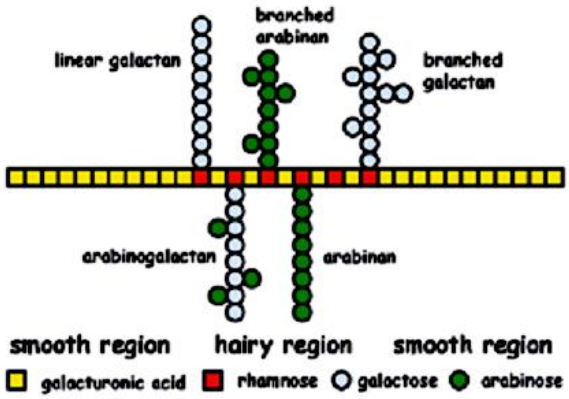

Figure 3. Schematic structure of pectin

Like galactose, galectins lie on the surface of your cells. By attaching to this sugar, galectins facilitate cellular communication, allowing your cells to relay messages to one another, and enabling them to stick together. This process is perfectly healthy in normal cells, where the number of surface galectins is relatively few.

Cancer cells, on the other hand, carry a disproportionate number of these galectins, specifically galectin-3, and this defining characteristic prove especially sinister. Hundreds of studies have pointed to the role of galectins in cancer development over the years the most recent have exposed galectin-3 as a key player in the growth and spread of cancer within the body.

Healthy cells die and regenerate as part of an orderly process as one becomes sick, another is produced to replace it. When this cell formation accelerates, however, it causes cells to "pile up" and form a tumor. But as long as these cells appear normal and static, the tumor is considered harmless, or "benign."

Unlike benign tumors, cancerous tumors are malignant. They're marked by uncontrolled growth and the ability to spread aggressively, a process known as metastasis. If given the opportunity, they will spread through your entire body, invading healthy tissues and causing new tumors. And this dangerous ability hinges on the presence of galectin-3.

Galectin-3 promotes cancer progression in three interconnected ways:

- It allows cancer cells to attach to one another, forming groups that can survive in your bloodstream and migrate to other parts of your body.
- Once cancer cells have formed a main tumor, galectins allow the cells to attach themselves to new sites as well, forming secondary tumors.
- Lastly, galectin-3 nourishes malignant tumors by stimulating new blood vessel to feed the tumor. This process is called angiogenesis.

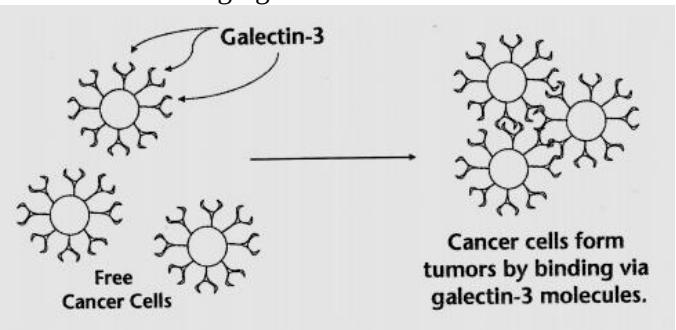

Figure 4. A schematic of the clumping of galectin3

It is no surprise, then, that these deadly galectins have become a primary target in modern cancer prevention. If you disarm a cancer cell's ability to communicate, you essentially pull the plug on its power supply it cannot spread or nourish itself, and ultimately, it will die. Pectin's molecular structure makes it a vital weapon in this inhibitory process by tying up galectins on cancer cells' surface, it can disable their ability to communicate with cells around them (Wang, 2002; Sharma, 2006).

It is stated that pectin that is efficient in binding the galectin-3 molecules is pectin having degree of esterification less than 50%. Degree of esterification is a measurement which dictates its ability to bind effectively to galectins. Esterification is when a galacturonic acid group along the pectin chain has an attached bulky methyl group. Simply put, the degree of esterification is the ratio of Galacturonic acid residues that are esterified, meaning having a methyl group attached to them compared to the ones that are free. 10 percent esterification means that one out of every 10 galactose molecules is bound and therefore not available for galectins to be able to bind to it. It also means that it can not bind to toxins or heavy metals that are positively charged.

Pectin extraction

Pectin extraction is a complex physicochemical process in which solubilization, extraction and depolymerization of pectin macromolecules from plant tissues may take place. High-esterified pectins are readily extracted by hot water, but low-esterified pectins usually are not readily extracted under such conditionsbecause they are physically bound in situ via metallic cations, especially divalent
cations. Sequestering agents, such as sodium hexametaphosphate, ammonium oxalate, ethylene diamine tetraacetate (EDTA) and cyclohexane diamine tetraacetate (CDTA), which readily bind cations, are added to the extractants for efficient extraction of this type of pectin. The presence of an acid or base and elevated temperature help cell wall disruption, protopectin hydrolysis and solubilization of pectic substances. In such processes, degradation of pectin macromolecules probably takes place. Extraction with dilute alkali generally yields a pectin with a low degree of esterification as a result of saponification of ester group, whereas the acid extraction yields a pectin with a relatively high DE (Wang, 2002).

Most commercial pectins are produced from apple pomace and citrus peels. The source materials are refluxed with hot dilute mineral acid at pH 2 and 60–100°C for 0.5–10 h. The extraction conditions are usually optimized with respect to yield, gelling capacity and desired DE. Fast-gelling pectins (DM >70%) are typically extracted at pH 2.5 and 100°C for 45 min; medium-, fast- and slow-set pectins (DM 60–70%) are extracted at lower temperatures for longer periods of time.

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To produce low-DE types of pectins, acid treatment is commonly applied to remove some of the ester groups in different stages of the extraction process. Alkaline hydrolysis can also be used for this purpose, but at a low temperature (<10°C) to prevent pectin degradation by β-elimination reaction. When ammonia is used for alkaline hydrolysis, a proportion of the ester groups is replaced by amide, and amidated pectins are obtained.

Enzymatic extraction of pectin

Pectins can also be extracted using enzymes. Scientific studies have all extracted pectins using galacturonase enzymes. This results in short but branched segments. In order to extract unaltered pectins arabinase and galactanase could be used to avoid degradation (Sharma, 2006).

Kabli and Garni (2006) enzymatically extracted pectin of grapefruit by using Kluyveromyces marxianus. Grapefruit waste contains about 37,5% pectin, 17,2% soluble sugars, and 14,3 holocellulose of its dry weight. The highest bioextraction of grapefruit pectin by using Kluyveromyces marxianus (356%), extracellular protein (5,5%) and protopectinase activity (12,02μ/ml), as well as, yeast growth (342.10^8 cfu/ml) were obtained under the following optimized fermentation condition: absence of yeast extract, 0,4% peptone, 1% glucose and 8% graperuit waste, under shaked conditions (200 shakes/min) for 18 h at 30^0C, pH 5 and seed culture of 24 h at 4% level.

Ptichkina et.al (2008) also extracted the pectin of pumkin with the aid of microbial enzyme. The enzyme was prepared from Aspergillus awamori. The characteristic of the pumpkin biopectin in comparison with acidic hydrolysis (0,1 N HCl) is shown on Table 1.

Table 1. The characteristic of the pumpkin biopectin in comparison with acidic hydrolysis

	Acid extraction	Enzymatic extraction with Aspergillus awamori
Hydrolysis time (h)	2	3
Yield (%)	7	14
Moisture content	9,2	9
pH of 1% solution	3,2	5,2
Polygalacturonate (%)	79	64
DE (%)	66	53
Molecular mass (kD)	70	45
Gel Strength (kPa)	31	10

The main action of the enzyme complex from A.awamori is to degrade cellulose and other insoluble constituents of the plant tissue, but it also has some pectinesterase activity, which could allow degree of esterification (DE) to be manipuated by varying digestion time. The time used in this investigation (3h) gave a DE of 53%; reduction in DE at longer times should yield pectin with a higher content of unesterified galacturonate residues, capable of binding lead and other heavy metal cations. Some possible medicinal and food uses are suggested for the pectin produced.

Many other studies have been conducted on the feasibility of utilizing enzymes for pectin extraction. One study used Trichoderma viride cellulase, Aspergillus niger hemicellulase, and a crude glycosidase complex from Xanthomonaas campestris to extract pectin from pumpkin pulp. The extraction conditions were a 3:50 dry solid to liquid ratio, 30CC, 20 hours, and 250 mg of hemicellulase, 50 mg of cellulase, or culture fluid of X. campestris. The data showed that there was a considerable increase in yield, from approximately 5% using acid extraction up to 22% using cellulase extraction, with cellulase producing the highest yield. The enzymatically extracted samples had low molecular weights with more difficulty in gelation (Campbell, 2006).

Another study was conducted in extracting pectin from citrus peel and apple pomace using polygalacturonase from Kluveromyces fragilis. Optimal extraction of pectin from citrus peel at yields of 16 to 20% of the dry matter was reported for conditions of a solid to liquid ratio of 1:12, 24 hours, 37CC, and 1.2 U enzyme activity. Another researcher reported that solid to liquid ratio, incubation period, age and size of microbial inoculum, and pH all influenced microbial extraction of pectin from beet. Solid to liquid ratio, extraction time, and extraction temperature were optimized in a study using Trichosporon penicillatum, which produces a protopectinase activity for

microbial pectin extraction from citrus peel. Optimal conditions were reported as a 1:2 solid to liquid ratio, 15 to 20 hours, and 30CC resulting in 2.5 g pectin per 100 g of peel (Campbell, 2006).

Research conducted by Panouile et.al (2007) showed that in pectin extraction with the aid of enzyme, the combination of a protease and a cellulase improves GalA solubilisation compared to separated use. With protease and cellulase, pectins are mostly extracted as polymers, contrary to results obtained with a pectinase.

Commercial enzymes are characterized by its expensive price. Many effort has been done in order to overcome this problems such as the re use of the enzyme and the utilization of enzyme isolated from local resources. Cellulose is an enzyme that can be isolated from various surces such as from fungus, bacteria, and ruminansia, while protease can be isolated from source such as pineaple or from calotropis gigantea (Murtini, 2003).

CONCLUSION

Premna oblongifolia Merr. is a potential source of low methoxyl pectin which is a potential candidate for anti cancer agent. The studies of many researchers indicate that a considerable increase in yield can be obtained by using enzymes for pectin extraction. But nowadays though enzymatics extraction can result in high yield and selectivity it has the main demerits of the enzymatic extraction which is the high price of commercial enzyme. In order to overcome all the demerits of pectin extraction, it is proposed to enzymatically extract the pectin of green cincau by using enzyme isolated from the local resources such as cellulase which can be isolated from hepatopancrease of snail and protease isolated from Calotropis gigantea.

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