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PRODUCTION OF LOW METHOXYL PECTIN AS AN ANTI CANCER AGENT FROM CITRUS PEEL PECTIN THROUGH ENZYME DEMETHYLATION BY PAPAYA PECTINESTERASE

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Abstract

Low methoxyl pectin (LMP) is reported posses anti cancer activity. LMP administering could reduce the risk of cancer, halt the progression of cancer, and in a certain percentage of cases caused the cancer cells to start to die. If this can be developed further, LMP administration could be a good co-treatment for chemotherapy or radiation. This would be a positive advancement, due to the high toxicity to the body of both chemotherapy and radiation. LMP can be produced by demethylation of high methoxyl pectin. One of our local resources that is potential for its pectin content is citrus peel. Pectin demethylation can be conducted by acid, alkali, ammonia in alcohol or enzymatic method. LMP produced by enzyme demethylation have been found to be inferior in quality to those produced by other methods. The enzyme took part in the pectin demethylation is pectinesterase. Pectinesterase can be be isolated from various source such as fruit and vegetables. One of our local resources that is potential as source of pectinesterase is papaya. Considering facts above thus it has a great possiblity to produce LMP from citrus peel pectin through enzymatic demethylation by utilize pectinesterase of papaya.

Key-word : Low methoxyl pectin pectin (LMP), anti cancer, citrus peel pectin, papaya, pectinesterase

INTRODUCTION

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. Citrus peel is one of raw material for pectin production that is vary potential to be developed further more in Indonesia because citrus is one of fruits with high economic value in Indonesia. The production of citrus increases from year to year due to national fruits development program. In 2006 the citrus production is up to 2,4 million ton (Ahmad, Mardison, Tjahyohutomo, & Nurhasanah, 2010). With the numerous amount of citrus production, it estimated that the potency of the citrus peel will be numerous too.

Citrus peel is known to be rich of pectin. Pectin is one of major part of plant cell wall. Plants cell walls 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, Pagan, & Shi, 2002).

Low methoxyl pectin are produced by further deesterification to a point where less than 50% of the total carboxyl groups are esterified. There are four

different methods for the preparation of low methoxyl pectin from high methoxyl pectin. HMP pectin demethylation could be affected by: (a) acid, (b) alkali, (c) enzyme and (d) ammonia in alcohol or concentrated aqueous ammonia demetylation and amidation (Alemzadeh, Saifkordi, Kahforooshan, & Nahid, 2005).

Acid demethylation removes special units at a high rate, leading to the production of pectin having a high percentage of poly galacturonic acid. The main disadvantage of acid treatments is the slowness of the reaction. It may be speeded up by using higher temperatur, but this result in the de polymerization of the pectin chain. Alkaline demethylation is rapid but removal of methyl ester groups is accompanied by the de polymerization of the pectin chains and the rate of the depolymerization is faster than the rate of demetylation as the temperature increase. The use of ammonia in alcoholic or concentrated aqueous ammonia system result in low methoxyl pectin that contains amide group, and it will affect the gelling properties (Alemzadeh, Saifkordi, Kahforooshan, & Nahid, 2005).

Low methoxyl pectin produced by enzyme demethylation have been found to be inferior in quality to those produced by other methods, because of the non random distribution of methyl ester groups among molecules of the pectin and because of the removal of very small units, such as non-uronide materials.

Enzyme demethylation can be conducted by using pectinesterase. Pectinestarase removes methoxyl groups from methylated galacturonic residues of pectic substances. This enzyme is widely distributed in higher plants and can be found in different plant tissues; mainly those contained in fruits such as tomato, orange , papaya, apple, kiwi, grapefruit pulp and mandarin orange fruit. From other plant sources, PE has been extracted and partially purified from potato and from seeds of Ficus awkeotsang (Saenz, Tellez, Garza, Reyes, Esquivel, &Aquilar, 2000).

Considering the health benefit of LMP and the inferiority of the enzyme demethylation of pectin, hence it is considered neccesary to present a paper that gave a description on citrus peel pectin, LMP mechanism in preventing cancer, enzyme demethylation of pectin, pectinesterase, and papaya pectinesterase.

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.

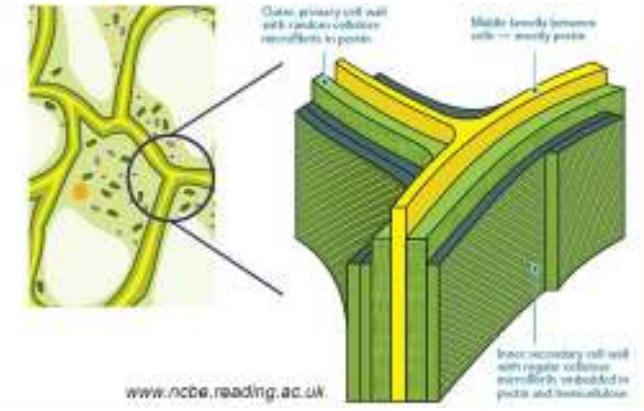

Figure 1. Schematic Representation of the Plant Cell Wall.

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 1). 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 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, Pagan, & Shi, 2002).

CITRUS PEEL PECTIN

The primary source of pectin is apple pomace, in which pectin is present in high amounts up to 20 grams per 100 grams of source material. Citrus peel, also a byproduct of juice production, is the other major source of industrial pectin, exhibiting extraction weights of approximately 20 grams per 100 grams of source material (Sundeen, 2009). Here, the parts that hold the most pectin are not the parts that contain juice, but instead the peel and the core (Figure 2).

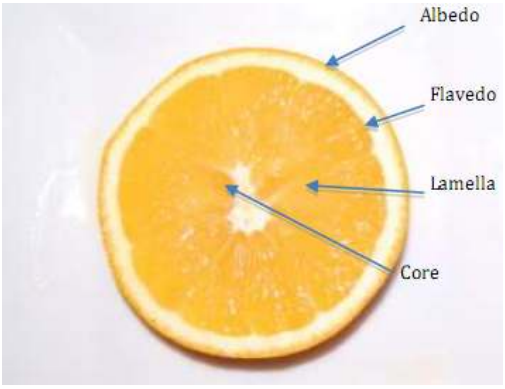

Figure 2. A picture of an orange, the portions where the most pectin is found.

The composition of pectin is differ one from another, it is depend on the source of the raw material of the pectin extraction. The composition of a number of pectin source such as pectin from sugarbeet pulp, apple pomace, citrus peel and pea hulls are shown on Table 1 (Sharma, Dhuldhoya, & Merchant, 2006).

Table 1. Composition of a number of pectin source

Component	Sugar beet pulp (%w/w)	Apple pomace (%w/w)	Citrus peel (%w/w)
Rhamnose	1,1	1,5	1,3
Arabinose	17,3	8	6,4
Xylose	1,5	5,5	2,4
Mannose	1,5	1,8	2,2
Galactose	4,3	5	3,2
Gc	21,7	27,9	19,6
Galacturonic Acid	18,9	25,2	26
Methanol	2,3	2,2	-
Ethanol	3,6	2	-
Proteins	8	5,7	-
Lignin	1,8	-	-
Ash	8,4	2	-

CITRUS PRODUCTION IN INDONESIA

Citrus is one of fruits with high economic value in Indonesia. The Government of Indonesia, through the Ministry of Agriculture, has a program to increase local citrus production to substitute import citrus, and later on to export high quality citrus to other countries.

The citrus production has been increasing from year to year since 1999, and in 2006, the volume of production was about five times of that in 1999. The increase of citrus production was achieved due to the extensification and intensification programs. The extensification program increased the harvest area of 25.2 thousand hectares in 1999 to 67.2 thousand hectares in 2006, while the intensification program was able to increase the production yield per hectare of citrus from 17.83 ton/ha in 1999 to 36.93 ton/ha in 2006. As a result, total production of citrus in Indonesia has increased from 449.5 thousand tons in 1999 to 2.5 million tons in 2006. The complete data was shown in Table 2 (Ahmad, Mardison, Tjahyohutomo, & Nurhasanah, 2010).

Table 2. Citrus Production in Indonesia

Year	Harvest Area (ha)	Production (ton)
1999	25210	449531
2000	37120	644052
2001	35367	691433
2002	47824	968132
2003	69139	1529824
2004	66071	1994760
2005	62578	2150219
2006	67152	2479852

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.

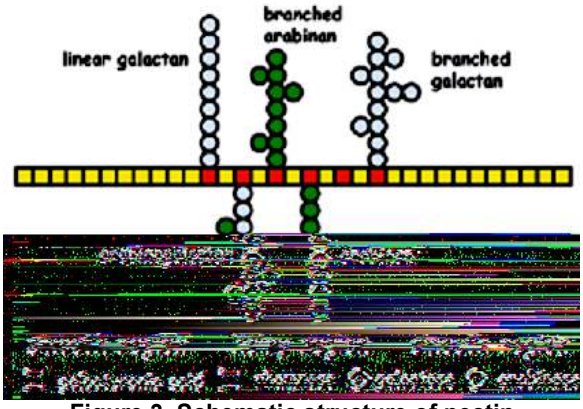

Figure 3. Schematic structure of pectin

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 are 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.

It is no surprise, then, that these deadly galectins have become a primary target in modern cancer prevention. If we can disarm a cancer cell's ability to communicate, we can essentially pull the plug on its power supply so it cannot spread or nourish itself, and ultimately, it will die. Molecular structure of pectin makes it as 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, Pagan, & Shi, 2002; Sharma, Dhuldhoya, & Merchant, 2006).

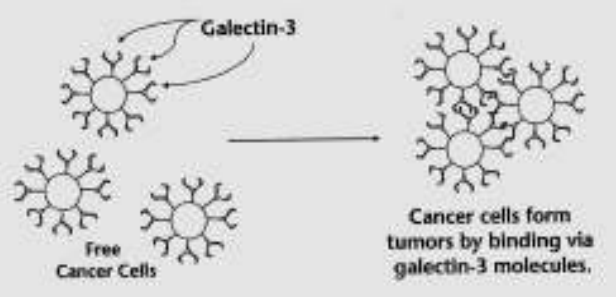

Figure 4. A schematic of the clumping of galectin3

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 DEMETHYLATION

Although some LMP occurs in plants, they are usually manufactured from HMP. Low methoxyl pectin are produced by further deesterification to a point where less than 50% of the total carboxyl groups are esterified. There are four different methods for the preparation of low methoxyl pectin from high methoxyl pectin. HMP pectin demethylation could be affected by: (a) acid, (b) alkali, (c) enzyme and (d) ammonia in alcohol or concentrated aqueous ammonia demetylation and amidation (Alemzadeh, Saifkordi, Kahforooshan, & Nahid, 2005).

Acid demethylation is commonly used to manufacture LMP. Acid demethylation removes special units at a high rate, leading to the production of pectin having a high percentage of poly galacturonic acid. The main disadvantage of acid treatments is the slowness of the reaction. It may be speeded up by using higher temperatur, but this result in the de polymerization of the pectin chain.

Alkaline demethylation is rapid but removal of methyl ester groups is accompanied by the de polymerization of the pectin chains and the rate of the depolymerization is faster than the rate of demetylation as the temperature increase. The usage of higher concentration of acid at low temperature gave less depolymerization during demethylation than when lower concentration and higher temperature were used (Constella & Lozano, 2003).

The use of ammonia in alcoholic or concentrated aqueous ammonia system result in low methoxyl pectin that contains amide group, and it will affect the gelling properties (Alemzadeh, Saifkordi, Kahforooshan, & Nahid, 2005).

Low methoxyl pectin produced by enzyme demethylation have been found to be inferior in quality to those produced by other methods, because of the non random distribution of methyl ester groups among molecules of the pectin and because of the removal of very small units, such as non-uronide materials.

PECTINESTERASE

Enzyme demethylation can be conducted by using pectinesterase. Pectinesterase (PE) is a ubiquitous cell-wall-associated enzyme that presents several isoforms that facilitate plant cell wall modification and subsequent breakdown. It is found in all higher plants as well as in some bacteria and fungi. Pectinesterase functions primarily by altering the localised pH of the cell wall resulting in alterations in cell wall integrity.

Pectinesterase catalyses the de-esterification of pectin into pectate and methanol. It removes methoxyl groups from methylated galacturonic residues of pectic substances (Figure 5) (Pedrolli, Monteiro, Gomes, & Carmona, 2009). In plants, pectinesterase plays an important role in cell wall metabolism during fruit ripening. In plant bacterial pathogens and in fungal pathogens, pectinesterase is involved in maceration and soft-rotting of plant tissue. Plant pectinesterases are regulated by pectinesterase inhibitors, which are ineffective against microbial enzymes.

Most of the purified plant pectinesterases have neutral or alkaline isoelectric points and are bound to the cell wall via electrostatic interactions. Pectinesterases can however display acidic isoelectric points as detected in soluble fractions of plant tissues. It has now been shown that some plant pectinesterase isoforms may exhibit both mechanisms and that such mechanisms are driven by alterations in pH. The optimal pH of higher plants is usually between pH 7 and pH 8 although the pH of pectinesterase from fungi and bacteria is usually much lower than this.

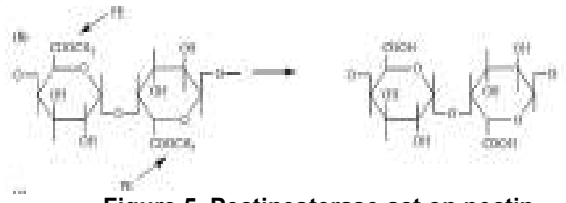

Figure 5. Pectinesterase act on pectin

PE is widely distributed in higher plants and can be found in different plant tissues; mainly those contained in fruits such as tomato, orange, papaya, apple, kiwi, grapefruit pulp and mandarin orange fruit. From other plant sources, PE has been extracted and partially purified from potato and from seeds of Ficus awkeotsang. Some reports established that plants contain multiple forms of PE differing in molecular weight, charge and glycosylation degree which affects the affinity for pectin and the thermostability of the PE forms (Saenz, Tellez, Garza, Reyes, Esquivel, &Aquilar, 2000).

PAPAYA AND PAPAYA PECTINESTERASE

Papaya is an edible melon-like fruit of a tropical softwood tree (Carica papaya) of the family Caricaceae. Today it is cultivated throughout the tropical world and in the warmest parts of the subtropics.

India stands first in the production of papaya in the world followed by Nigeria, Indonesia, Mexico, Ethiopia and others as seen in Table 3 (Varmudy, 2011).

Table 3. Production of Papaya in the World in 2008-2009

Country	Production (MT)	Percent of Share in World Production
India	3629000	36,1
Brazil	1900000	18,9
Nigeria	765000	7,6
Indonesia	653276	6,5
Mexico	638237	6,4
Ethiopia	260000	2,6
Congo	223770	2,2
Colombia	207698	2,1
Guatemala	184530	1,8
Philipines	182907	1,8

Papaya is considered one of the most economically important and nutritious fruits, being a rich source of antioxidant nutrients; the B vitamins folate and pantothenic acid; the minerals potassium and magnesium; and fiber. In addition, papaya is the source of pectines terase (EC. 3.1.1.11, a pectic enzyme that can have a great impact in te fruit and vegetable processing technology because of it s potential effect on the quality of the finished products.

Studies carried out by Ashraf (1993) showed that the incubation time, pH and NaCl concentration influenced the extraction of the enzyme from the papaya fruit. A maximum activity of 7.0 pmole of carboxyl groups/minlml (7.0 unit s/ml) was obtained when 2M NaCl solution (pH 8) and an incubation time of five hours were used. The procedure adopted for purification resulted in a 250-fold purification (784 unit s/mg protein) with a 45% recovery of the enzyme activity. The enzyme has an apparent molecular weight of approximately 32,000 Daltons. The purified enzyme was further characterised as a function of NaCl concentration, pH and temperature. Its kinetic properties were also studied. The activity was found to be linear up to 20 minutes with an enzyme concentration of up to 6.42 pg protein. Maximum activity was obtained using 0.25M NaCl solution, pH 8.0 and 65°C.

pH stability studies showed that the enzyme was stable from pH 4- 11 afer exposure of the enzyme to these pH for 24 hours at 30°C. More than 85% of the activity was retained in all of these cases . However, at pH 1 and 12, the enzyme was unstable and it completely lost its activity after 24 hours of in cubation at 30°C.

CONCLUSION

Low methoxyl pectin is a potential anti cancer agent. Pectin molecules is able to bond with a particular protein called galectin-3. Galectin-3 is a protein that has been recognized as a cancer-causing agent. Low methoxyl pectin is usually manufactured from HMP such as from citrus peel pectin. There are four different methods for the preparation of low methoxyl pectin from high methoxyl pectin. HMP pectin demethylation could be affected by: (a) acid, (b) alkali, (c) enzyme and (d) ammonia in alcohol or concentrated aqueous ammonia demetylation and amidation. Low methoxyl pectin produced by enzyme demethylation have been found to be inferior in quality to those produced by other methods. The enzyme used in the demethylation process is pectinesterase that can be isolated from various kind of fruit and vegetables such as from papaya.

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