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Research Article

ACIDIC METHOD FOR THE LOW MOLECULAR PECTIN PREPARATION

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ABSTRACT

Pectins are natural polysaccharides exerting various beneficial effects for human health and being considered as the prospective source of pharmaceutical agents. Due to the high molecular weight pectins cannot be absorbed in intestine and their influence is caused by the local activity in gastrointestinal tract. The method of acid pectin degradation allows preparation of pectin substances with low molecular weight making intestinal absorption of these compounds possible. With the use of this method pectin fractions with any necessary molecular-mass distribution can be obtained. Besides reduction of the molecular weight acid degradation is associated with de-esterification of their polysaccharide molecules. The velocity of the pectin degradation is highest during the first 30 min of incubation, then the rate of reaction decelerates and after 90 minutes of incubation remains constantly slow. During the first 90 minutes of incubation generally the neutral sugars presenting in pectin molecule are degraded whereas during the rest of incubation anhydrogalacturonic acid is slowly breaking. The results of the study describe the best conditions for preparation of the pectinswith low molecular weight.

Keywords: Low molecular pectin, Molecular-mass distribution, Method, Acid hydrolysis

INTRODUCTION

Pectins are commonly used in food industry and considered as prospective substances with beneficial health influence in humans due to their pronounced binding and water-holding capacity[1]. Also pectins are ideal materials for pharmaceutical application due to their rheological properties[2]. However, their effects are exerted in the space of gastrointestinal tract only because high molecular weight of the pectin molecule prevents it to be absorbed into blood. So, it is supposed that the usage of low molecular pectin substances would be more beneficial if these substances could be easily absorbed into blood and thus exert their influence on inner organs and systems in human body. It was shown previously that low molecular pectin compounds significantly accelerate Pb^{2+} removal with urine[3]. Besides, due to the low degree of polymerization modified low molecular pectins exert antitumor effects regarding colon cancer, breast cancer, myeloma and hemangiosarcoma[4-9]. This type of efficacy is provided by the presence of β-galactose residues in the pectin molecule. β-galactose is known to have binding properties regarding galectin-3, protein which responsible for the tumor growth and metastasis[10].

Generally low molecular pectins are prepared from the natural high molecular compounds using methods of chemical enzymatic degradation. This method is not convenient because it results in formation of very small oligogalacturonans containing just a few of uronic block capable to form contact zones[11]. At the same time acidic method of pectin degradation may be considered as the universal method for the low molecular pectin preparation, which can be executed with the use of mineral acids. This method opens a wide field for the discovery of effective and safe pharmaceuticals opposing the innovation deficit in the drug discovery process[12].

This work is devoted to the investigation of the acidic degradation processes of low and high esterified pectins with following estimation of the by-products contents. The main goal of this work was to establish the basic conditions for preparation of the pectin with different molecular weight which can be then used for development of the new pharmaceutical agents.

MATERIALS AND METHODS

Pectin samples

High-esterified citrus pectin without additives was obtained from Herbstreith& Fox KG, Germany. The stated degree of esterification of this preparation was 60.0%, the contents of anhydrogalacturonic acid was 58%. The pectin preparation contained no acetyl or amide groups. All other chemicals were of the highest quality available. Distilled water was used throughout the whole experiment.

Low-esterified pectin in a form of pectic acid was obtained from the high-esterified citrus pectin sample using the method of alkali de-esterification[13]. Degree of esterification of the low-esterified pectin sample was 1.3%, anhydrogalacturonic acid content – 78.8%.

Degree of esterification and anhydrogalacturonic acid content of the pectin samples were determined using titrimetric method[14]. Sedimentation of the pectin in solutions was performed by addition of double volume of 96% ethanol solution.

Pectin degradation method

Degradation of all pectin samples used in the study was performed under the same conditions with constant temperature of solution 90±0.5ºC. 0.5 M HCl was added for initialization of the molecule degradation process with the following constant stirring. Concentration of the high-esterified pectin in the reaction system was 1%; concentration of the low-esterified pectin used in form of suspension was 5%. The total volume of the pectin solution/suspension was 300 ml.

Within strictly determined periods of agitation the samples of degraded pectin were collected from the reactive system for chemical analysis. The volume of the samples collected varied from 5 to 15 ml. Deceleration of the degradation process was achieved by lowering of the reactive system temperature until 0ºC with ice added into water-bath. Liquid pectin phase was separated using centrifuging at 4000 rpm for 30 min. Removal of the soluble products of pectin degradation was performed by rinsing the samples with 0.5 M HCl and triple suspending with ten-fold acid volume following with centrifuging and liquid phase removal.

Estimation of the changes in degradation product content in dependence on the duration of reaction was performed using the method of step-by-step degradation. 30 ml of the pectin suspension was used. Agitation period of each stage of degradation was 30 min. After each stage of degradation the process was stopped, pectin liquid phase removed and contents of neutral sugar and anhydrogalacturonic acid in it determined. Pectin precipitation then was rinsed, suspended in 30 ml of 0.5 HCl and the next stage of degradation process was started.

Before investigation of the second stage of the pectin degradation process the samples were degraded for 1.5 h. Then pectin precipitate was rinsed for removal of the soluble by-products and re-suspended in initial volume of 0.5 M HCl. Fraction contents of the

liquid pectin phase after the second stage of degradation were estimated using ultrafiltration membranes. Before the degradation concentration of the pectin was assessed by separation of the pectin precipitate with the following drying at 100ºC until its mass does not change. Anhydrogalacturonic acid content in solution was determined by reaction with m-hydroxyphenyl;D-galacturonic acid was used as a standard[15].

The total carbohydrate content of each sample was calculated according to the phenol-sulfuric method with D-galactose used as a standard[16]. Calculation of the neutral sugar content was performed by taking anhydrogalacturonic acid amount determined by reaction with m-hydroxyohenyl from the total carbohydrate content.

Molecular-mass distribution of pectin degradation products was estimated according to the fraction composition determined with the use of ultrafiltration membranes with different limits of penetration. It was performed by separation of 10 ml of the pectin liquid phase with the following addition of 1.0 ml 0.2 M sodium citrate and neutralization (until pH 4.0) with 5 M NaOH. After that the total volume of solution was made up to 50 ml by addition of distilled water. Degraded pectin solution was fractioned using cellulose ultrafiltration membranes with the penetration limits 10, 3 and 1 kD under the pressure 0.25-0.3 MPa.

Anhydrogalacturonic acid content was determined in both, initial and final supernatant. The difference between both concentrations was used for calculation of the corresponding fractions of galacturonic acid.

Composition of the pectin fractions in the precipitate was determined by the similar method. 15 ml volume of the pectin sample was centrifuged, the precipitate obtained was rinsed and suspended in 100 ml of distilled water with the following addition of 8.0 ml 0.2 M sodium citrate solution and neutralization (until pH 4.0) with 5 M NaOH. Then the final volume of solution was made up to 400 ml with distilled water.

RESULTS AND DISCISSUION

It was figured out that degradation of the high-esterified pectin molecules was associated with hydrolysis of glycoside links as well as break out of the ether bonds in methylated carboxyl residues of the galacturonic acid blocks. Therefore, degree of esterification of the initial pectin samples lowered from 58% to 25-27% within the first 20 min of degradation (Fig. 1).

According to the reduction of the degree of esterification the solubility of the pectin in acid solution was also decreasing. Reduction of the degree of esterification to 40-35%, which was achieved in approximately 15 min since the start of the process, resulted in insoluble pectin gel particles formation. This process took place within 15-25 min of the degradation process and finished when degree of esterification achieved 20%. At this point the whole mass of the high molecular pectin was precipitated in a form of voluminous sediment. Therefore, consequent degradation process took place as a heterophase reaction whereas liquid phase contained the soluble products only. At this point a high-esterified pectin degradation process virtually became the low-esterified pectin degradation.

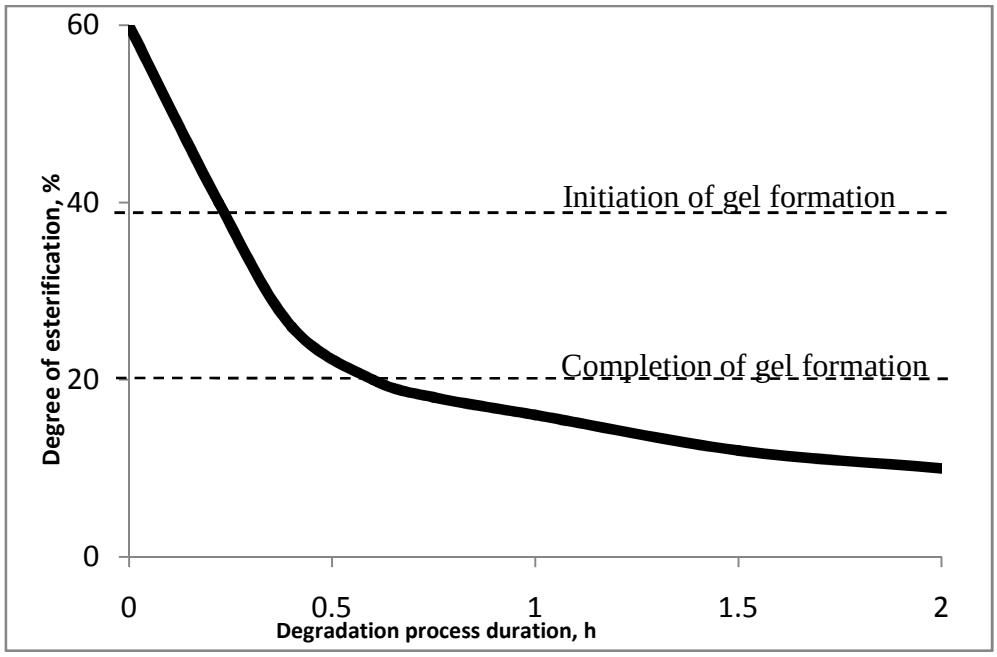

Fig. 1: Degree of esterification of the pectin molecule during the process of acidic degradation. Parameters of the reactive media: high-esterified pectin concentration 1%, HCl concentration – 0.5 M, temperature - 95±0.5ºC

In contrast to the high-esterified pectin the low-esterified sample being in the acid media instantly formed a solid gel containing up to 25-28% of dry substances and the degradation process from the very beginning was a heterophase reaction as follows: high-molecular pectin gel – acid solution.

Different conditions influencing the pectin degradation process were modeled using pectin samples with different degrees of degradation including initial low-esterified pectin and its derivatives preliminary subjected to hydrolysis of various durations. Soluble products were removed by rinsing with 0.5 HCl. Duration of the degradation periods did not exceed 20 min because it prevented possible degradation and lowering of the molecular weight of the pectin molecules transferred into solution. It was figured out that the amount of the molecules with their weight less than 10 kD in the liquid phase was 66-72% in comparison with the initial pectin sample and 75-78% regarding the sample initially degraded for 10 h. At the same time amount of such molecules in the gel phase was only 2.5% and 4.6% respectively.

Degradation velocity was estimated in accordance with accumulation of the low molecular products in the liquid phase during degradation of the 5%low-esterified pectin suspension and 1% high-esterified pectin solution. Calculations were carried out using determination of the anhydrogalacturonic acid content in the liquid phase within the same periods of reaction (Fig. 2). The plot of the anhydrogalacturonic acid concentration changes expressed in per cent is shown on the figure 3. 100% velocity of the process means highest change of the anhydrogalacturonic acid content within the fixed period of reaction.

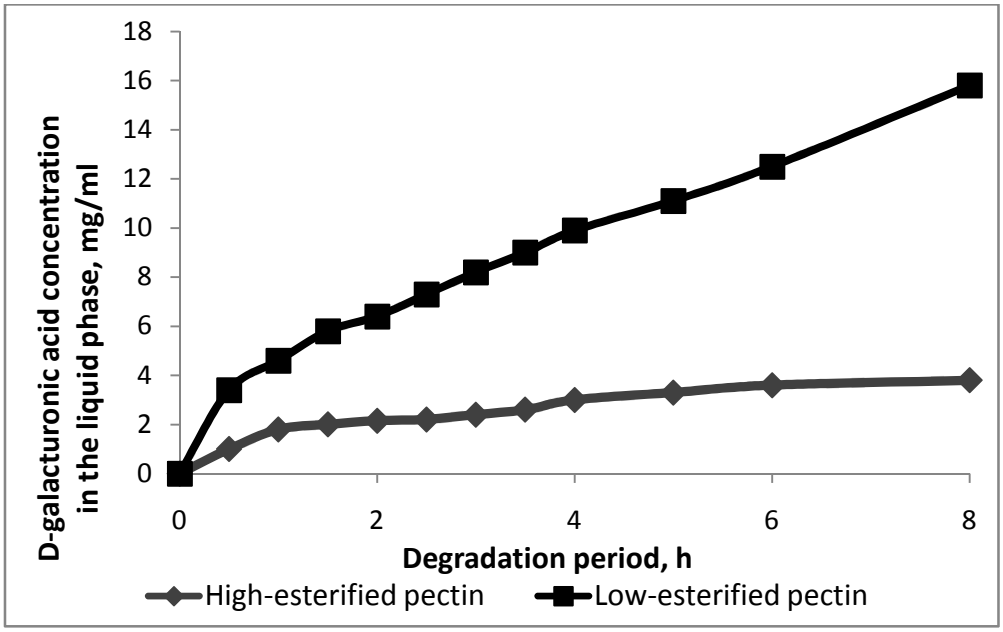

Fig. 2: Concentration of anhydrogalacturnic acid in liquid phase after various periods of incubation

Process of the pectin hydrolysis may be divided into two stages. At the very beginning of the process its velocity is the highest, therefore, accumulation of the low molecular products containing anhydrogalacturonic acid in the liquid phase is relatively fast. Then the dramatic decrease of the process speed is noted within the first 40-50 min of the reaction and the rate of the soluble product accumulation in liquid phase reduced in three-fold. The next stage of the degradation process is characterized by the slow changes of the process velocity. During 6 h of the reaction its speed slowed by 25%.

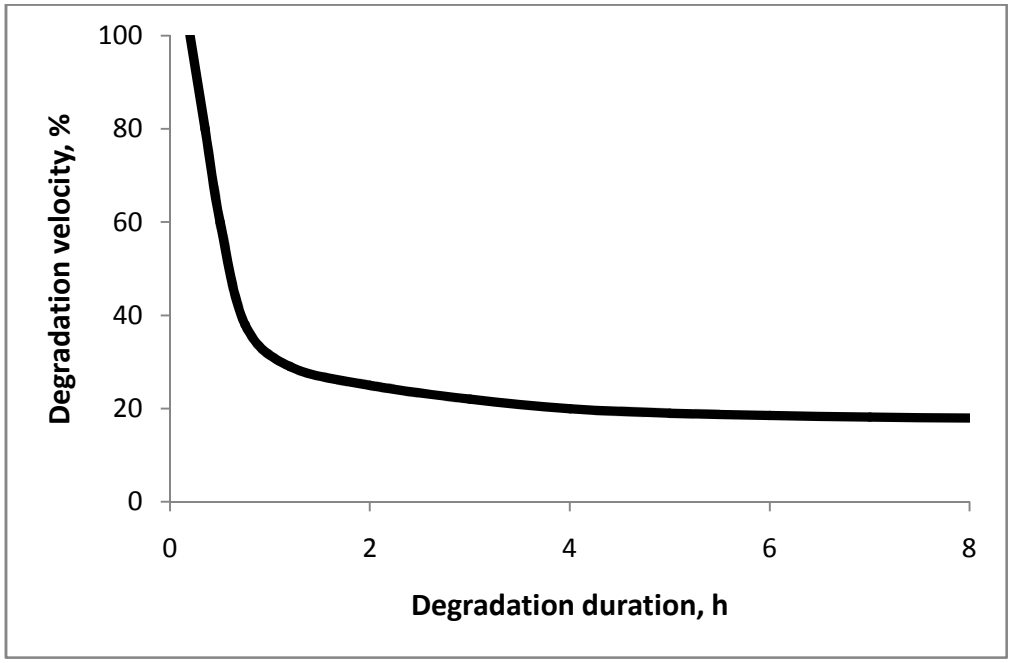

Fig. 3: Velocity of the pectin degradation process

Investigation of the degradation product contents in the liquid phase was performed using step-by-step hydrolysis. At the beginning of the process the relative content of anhydrogalacturonic acid in liquid phase gradually increased from 37.2% to 94.4% and then remained constant. Relative amount of the neutral sugars in the liquid phase consequently decreased from 62.8% to 5.6%. Therefore, it may be concluded that degradation of the major part of neutral sugars was happened within the first 90 min of the degradation process.

Thus, during the first 90 min stage of the pectin degradation the hydrolysis of the polysaccharide molecule parts containing neutral monosaccharaides and easily broken anhydrogalacturonic acid occurs. After this period the total amount of the low molecular products transferred into the liquid phase was 23.3% from the total pectin mass. The part of the neutral sugars in this phase was 17.1% i.e. 80.5% from their initial content in the pectin. The part of anhydrogalacturonic acid respectively was 6.2% meaning 7.8% of its share in the initial pectin sample.

Second stage of the pectin degradation process was studied separately using the pectin sample preliminary subjected to the first stage of degradation during 90 min. The general conditions of the reaction did not change, therefore pectin concentration in suspension reduced from 5% to 3.85% as a result of the first stage degradation process.

Second stage of the degradation is associated with hydrolysis of almost pure polygalacturonic acid and small amount (5.6%) of neutral sugars. Process was characterized by virtually unchanged velocity.

During continuing 10-hour degradation process the amount of all low molecular pectin fractions except the one of 10 kD in liquid phase gradually increased. Amount of the 10 kD pectin fraction was 0.305 mg/ml after 30 minutes of incubation, and then within 4.5 h the two-fold increase of its concentration was noted achieving 0.600 mg/ml. After next 5 h of incubation concentration of the 10 kD pectin fraction increased only by 8% making up to 0.650 mg/ml. Increase of other pectin fraction during the degradation process was more dramatic. From 1 h to 10 h of degradation the amount of 3-10kD pectin fraction increased in 4.15 fold from 0.32 to 1.33 mg/ml, amount of 1-3 kD fraction increased in 5.4-fold from 0.093 to 0.502 mg/ml, amount of the less than 1 kD pectin fraction increased in 9.0 fold from 0.22 to 1.98 mg/ml. Changes of the difference of pectin fraction ratio expressed in per cent during the process degradation were as follows. Fractions more than 10 kD : fraction 3-10 kD : fraction 1-3 kD : fraction less than 1 kD were corresponding at the first 30 min of incubation as 44.0 : 37.0 : 6.3 : 12.7. After 2 h of incubation this ratio was 28.5 : 31.5 : 10.0 : 30.0. At the end of the process after 10 h of incubation this ratio was 14.5 : 30.5 : 10.5 : 44.5 respectively (Fig. 4).

Structural characteristics of pectins are typical of complicated heteropolysaccharides. Their structure contains linear homogalacturonan blocks consisting of D-galacturonic acid as well as branched blocks of rhamnogalacturonan containing large amounts of neutral monosaccharides[17]. These monosaccharides being the part of pectin molecules are linked each other via glycoside bonds possessing various resistance to the damaging influence of acids. This explains significant differences in velocity of degradation process and in composition of the degraded products obtained after different stages of the process. Degradation begins with the most instable bonds to be broken which are generally formed from the neutral sugars. Ratio between neutral sugar content and amount of anhydrogalacturonic acid after the first 90 min of degradation process is 2.76 : 1 which is well corresponding to the known rhamnogalacturonan structure[18]. The remaining part of the pectin which is hardly hydrolyzed consists generally from the anhydrogalacturonic acid being obviously a high molecular polygalacturonic acid (homogalacturonan). According to the previously obtained data glycoside bonds formed by uronic acids are very resistant to the acid hydrolysis[19]. Ratio between fractions in the liquid phase after the second stage of the degradation process is equally shifted towards low molecular compounds. Managing the degradation period makes possible to obtain the final products enriched with necessary molecular fraction of polygalacturonic acid.

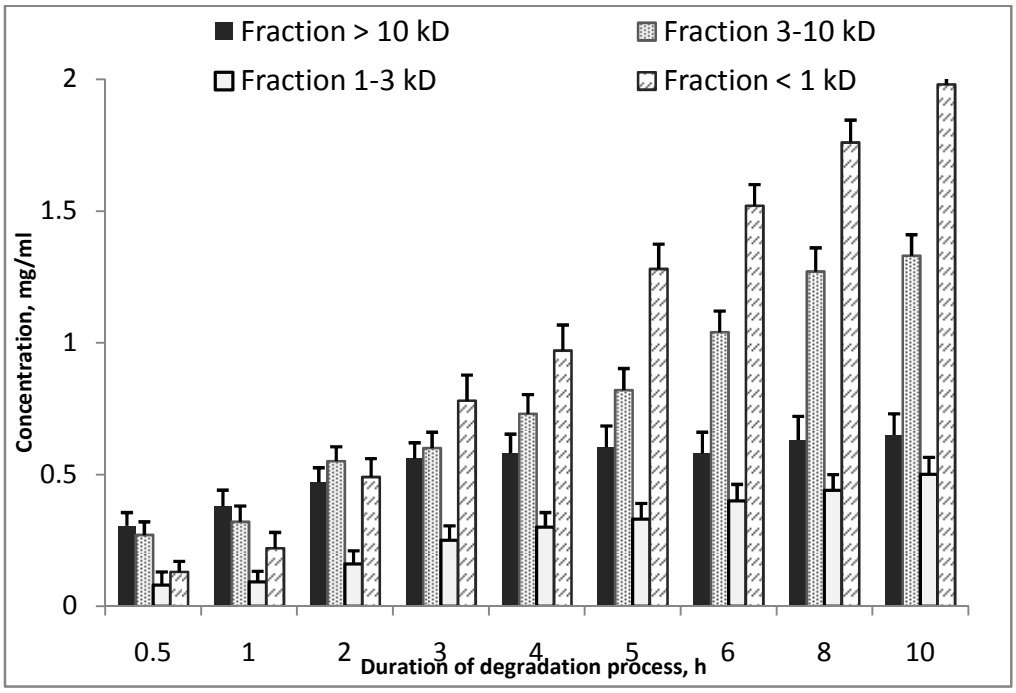

Fig. 4: Concentration of the low molecular pectin fractions in liquid phase depending on the incubation period

Thus, method of acid hydrolysis may be successfully used for obtaining of the low molecular fractions enriched with neutral sugars as well as fraction containing anhydrogalacturonic acid only. Use of the low esterified pectin makes possible to increase the rate of the degradation process by enhanced pectin concentration and makes separation of low molecular and high molecular fraction of pectin much simpler by the means of natural phase transfer "gel – solution".

CONCLUSION

Acidic method of low molecular pectin preparation may be considered as the universal one for obtaining the fractions of pectin with any necessary molecular weight. Pectins with molecular weight less than 5 kD may be used for development of the new pharmaceutical spossessing beneficial properties of natural pectin compounds exerting effects caused by their distribution in the bloodstream and systemic influence on the body. Thus, this method discovers a prospective to create the new class of the safe and effective pharmaceuticals.

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