HUNGARIAN JOURNAL
OF INDUSTRIAL CHEMISTRY
VESZPRÉM
Vol. 36(1-2) pp. 55-58 (2008)

KINETIC STUDY ON HYDROLYSIS OF VARIOUS PECTINS BY *ASPERGILLUS NIGER* POLYGALACTURONASE

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Hydrolysis of pectins from various sources by a polygalacturonase (PG) enzyme was studied from a kinetic point of view. Pectin substrates – which are commercially not available – were extracted from sugar beet pulp, apple, red currant and black currant. Strong product inhibition was found in each pectin preparations that could be described by a competitive mechanism. The kinetic parameters (Michaelis-Menten constants, maximal reaction rates and inhibition constants) were determined and compared. Differences in the parameters imply distinctions in structure of the pectins studied.

Keywords: product inhibition, enzymatic hydrolysis, galacturonic acid,

Introduction

In the fruit-processing industry pectolytic enzymes are used to increase yields, improve liquefaction and clarification [1,2], moreover to produce D-galacturonic acid (monomer of pectin), which is an important compound, raw material in the food, pharmaceutical and cosmetic industry to manufacture e.g. vitamin C, or acidifying, tensioactive agents [3]. Hydrolysis of pectin can be carried out by pectinases that are classified into three main groups:

- pectinesterases – catalysing deesterification of the methoxyl group of pectin;
- depolymerising hydrolytic enzymes (including polymethyl-galacturonases and polygalacturonases) – catalysing the hydrolytic cleavage of 1,4-glycosidic bonds;
- lyases – catalysing the cleavage of glycosidic bond by transelimination.

Among the pectin hydrolysing enzymes (endo)poly-galacturonases are probably the most important biocatalysts. Polygalacturonase enzymes (PG, E.C. 3.2.1.15.) are able to hydrolyse pectin and/or pectic acid. Although PG enzymes play a key role in pectin hydrolysis, their actions have not been studied in details from kinetics point of view so far, while kinetic behaviour of many soluble and immobilized pectinase enzymes and enzyme-mixtures have been already characterised [4,5,6]. Kulbe et al. [7] have assumed that the PG enzyme from *Aspergillus niger* was inhibited by its monomer, but no experiments were carried out to prove it and determine the mechanism.

We have studied the kinetics of pectin hydrolysis by polygalacturonase enzyme from *Aspergillus niger* in details using a commercially available, low esterification degree LM-5CS pectin substrate and the kinetic parameters were determined [8]. The aim of this work is to study the kinetics of enzymatic hydrolysis of other pectins, which are commercially not available. These pectins, therefore, should be prepared in our laboratory by extraction from plant substances containing considerable amount of pectin.

Materials and methods

Polygalacturonase enzyme (PG) from *Aspergillus niger* was purchased from Sigma (USA), its activity was 1.7 U/mg. Activity definition: one unit is defined as the amount of enzyme which is able to produce 1 μmol galacturonic acid from polygalacturonic acid in one minute in pH = 4.1 and 50 °C. The enzyme is able to hydrolyse pectin molecules, as well. All the other chemicals (analytical grade) were purchased from Fluka (Germany).

Pectin substrates were extracted from sugar beet pulp, apple, red currant and black currant by boiling water [9]. The substance-water mass ratio was 1:4, the extraction time was 4 hour. As a result, majority of the pectin was obtained in the aqueous phase. Ultrafiltration was used then to clarify the extracted solution, and the diluted aqueous pectin solution was concentrated partly by membranes (ultrafiltration, polyethersulfone membrane, cut off 45 kDa) up to 5 % TSS, partly by evaporation up to 30 % TSS. Then pectin in powder form was obtained

from the concentrated pectin solution after precipitation with alcohol.

Pectin purity was determined by HPLC (Merck system) equipped with BioRad Aminex HPX42-A column and RI detector, ultrapure water was used a mobile phase. Purified citrus pectin was used as a standard for comparison.

In the kinetic experiments purified apple and sugar beet pectins were used to compare to the other pectin preparations.

To study the kinetics of the reaction, shaking flask experiments (three parallel each) were carried out in a New Brunswick Scientific (USA) shaking incubator. Citrate buffer was used (pH = 4.1) to prepare the substrate solutions with various concentrations, and the operational conditions of the experiments were 50 °C and 150 rpm. To determine the product inhibition, galacturonic acid (product) was added initially to some of the substrate solutions.

The hydrolytic reaction was followed by measuring the reducing sugar content using the dinitro-salicylic test (DNS standard method – Miller (1959) [10]) which is based on the formation of a chromophore between DNS product and reducing groups of the (oligo)galacturonic acid molecules.

Degree of esterification was determined by the titration method [11] involving the titration of the pectin suspension with sodium hydroxide before and after the saponification step.

In the de-esterification procedure the carboxyl groups of the pectin were hydrolyzed, while the pH of the pectin solutions was maintained at 12, using 0.1 M NaOH. After saponification 0.1 M citric acid was added to the mixture to adjust the pH at 4.1.

Results

Pectin extraction

Pectin substrates were extracted from various sources, the average yields obtained were summarized in *Table 1,* and data on original pectin content [12] of the fresh substances are presented for comparison, as well. It can be seen that majority of the pectin content was successfully recovered from the tissues. The average purity of the various pectin preparations was determined by HPLC and the initial substrate solutions for the kinetic study were prepared taking into account the exact pectin content of the preparations.

Table 1: Pectin yields and purity

Substance	Yield of extraction [%]	Purity [%]	Original pectin content [%]
Apple	0.93	82.9	1.5-1.6 [2]
Red currant	0.95	85.4	1.2-1.4 [13]
Black currant	1.25	90.6	1.4 [12]
Sugar beet	11.35	93.4	13.1[2]

Study on the kinetics

In the shaking flask experiments firstly progress curves on hydrolysis of pectin solutions were measured, where the reducing sugar contents (galacturonic acid) in the reaction mixtures as a function of time were determined with different initial substrate concentrations. As an example, experimental data on various initial substrate concentration (using 0.01 g enzyme) for red currant pectin preparations are presented in *Fig. 1.*

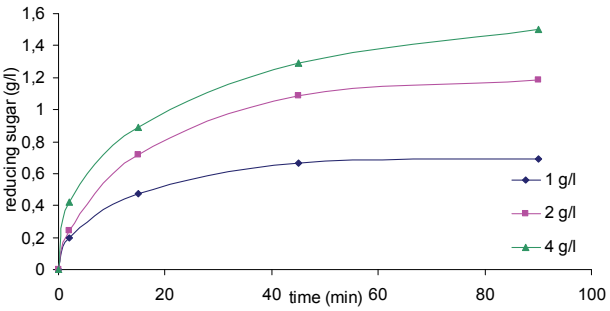

Figure 1: Progress curves of pectin hydrolysis by polygalacturonase from Aspergillus niger (Reaction conditions: pH 4.1, 150 rpm, 50 °C, 0.01 g enzyme, red currant pectin)

Since earlier measurements for citrus pectin have proven that product inhibition occurs during the reaction, another series of experiments were carried out to study the effect of the product on the process. Various amounts of galacturonic acid were added initially to the reaction mixture using different substrate concentrations and the reducing sugar content was measured as a function of time *(Fig. 2).* In Fig. 2 it is clearly shown that the galacturonic acid present had a significant inhibition effect on the reaction. The more product to the reaction mixture was added, the slower initial reaction rate was observed.

Investigating the hydrolysis of pectins from different sources similar progress curves were obtained. From the experimental data (progress curves) initial reaction rates were calculated which were then transformed according to Lineweaver-Burk method (double reciprocal method). The 1/v intercepts of all the lines were in the same section, thus it can be concluded that the type of inhibition was competitive.

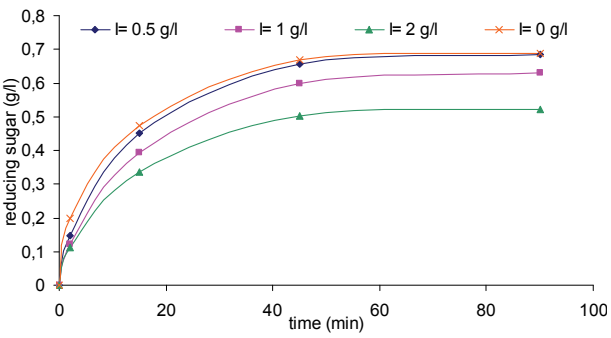

Figure 2: Example for progress curves on studying the product inhibition (Reaction conditions: pH 4.1, 150 rpm, 50 °C, 2 g/l red currant pectin, 0.01 g enzyme)

The Michaelis-Menten model for the reaction rate completed with competitive product inhibition describes the process as follows:

$$v_i = \frac{v_{max}(S)}{K_m \frac{K_i + (I)}{K_i} + (S)}$$

where

v_i	reaction rate
S	substrate concentration
I	inhibitor (product) concentration
v_{max}	maximal reaction rate
K_m	Michaelis-Menten constant
K_I	inhibition constant

Applying the Lineweaver-Burk method, the parameters of the model were determined (which were checked by numerical methods, as well) and their statistical analysis was carried out by SigmaStat program. The parameters obtained are summarised in *Table 2*.

Table 2: Kinetic parameters obtained for hydrolysis of various pectin preparations by PG from Aspergillus niger

Pectin	K_m [g/l]	v_{max} [g/l*min]	K_I [g/l]
Sugar beet	1.47	0.31	1.16
Sugar beet* [14]	3.0	0.43	0.16
Citrus	8.3	1.06	3.13
Citrus*[14]	3.5	0.23	1.05
Red currant	0.48	0.19	0.88
Red currant (after de-esterification)	0.48	0.47	0.93
Black currant	0.79	0.31	0.94
Black currant (after de-esterification)	0.95	0.82	1.04
Apple	0.15	0.08	0.58

Firstly the kinetic parameters of the pectins were compared with data found in literature [14]. These data are marked by asterisk. It can be concluded that the value of the data are in one order of magnitude. However full comparison is not possible through another enzyme preparation was used during the reactions.

It also implies – regarding pectins from other sources (including citrus pectin studied and described in our earlier paper) – that the considerable differences existing in the parameters for various pectins might be caused by the structural differences between the pectins. It is known that pectins from citrus fruits have low esterification degree, while sugar beet pectin contains not only large amount of methanol (esterified), but acetic acids, as well, bound to the backbone [9]. Pectins from berry fruits less information is available on the structure of pectins, therefore a study on the structure details should be accomplished. Nevertheless higher initial reaction rate was observed in case of de-esterified pectins compared to natural pectins.

Summary

Hydrolysis of various pectin preparations by polygalacturonase from *Aspergillus niger* was studied in details. It was found that strong product inhibition occurred during the process. The product, galacturonic acid is a competitive inhibitor of the enzyme, and the kinetic parameters (including inhibition constants) were determined experimentally as a new finding for each pectin substrates. Considerable differences in the constants were found for the various pectins implying significant differences in pectin structure.

Now our aims are (i) to explore and determine structural differences in pectin from various sources by special analytical methods, instrumental analysis (e.g. NMR, SEM, MS…etc.) and (ii) to extend the research work for other, cheaper substrates, like agro-waste material e.g. press cake formed in fruit juice production. Thus the processing of pectin containing fruits can be completed with a waste utilization step: manufacturing a valuable product, D-galacturonic acid by pectin extraction followed by enzymatic degradation. To avoid inhibition, membrane bioreactor should be applied for continuous pectin hydrolysis to enhance productivity. Finally the galacturonic acid can be recovered and concentrated by electrodialysis, to study and characterize this process is also one (iii) of our aims in this project.

ACKNOWLEDGEMENTS

The research work was supported by GAK (MEMBRAN5) project, grant No. OMFB-00971/2005. and GVOP Project, grant No. 0421/3.0.

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