Biochemistry (Moscow), Vol. 67, No. 6, 2002, pp. 676-682. Translated from Biokhimiya, Vol. 67, No. 6, 2002, pp. 815-822.

Viscometric Method for Assaying of Total Endodepolymerase Activity of Pectinases

A. V. Gusakov*, A. V. Markov, S. G. Grishutin, M. V. Semenova, E. G. Kondratyeva, and A. P. Sinitsyn

School of Chemistry, Lomonosov Moscow State University, Moscow, 119992 Russia; fax: (095) 939-0997; E-mail: avgusakov@enzyme.chem.msu.ru

Received April 16, 2001

Abstract—An improved method for assaying of the total endodepolymerase activity of pectinases has been developed. The method is based on the determination of the viscosity of a citrus pectin solution in the presence of the enzyme using an Ostwald viscometer. The depolymerizing activity of different pectinases can be detected including polygalacturonase, polymethylgalacturonase, pectin lyase, and pectate lyase. One unit of the endodepolymerase activity corresponds to the activity resulting in 50% decrease in the relative viscosity of 0.5% citrus pectin solution for 5 min at 40°C and the appropriate pH. Depending on the pH-optima of the enzymes, two modifications of the method are described: 1) for acid pectinases at pH 5.0, and 2) for neutral (mildly alkaline) pectinases at pH 8.0. The modifications differed in the control and in the calculation of the activity. Six enzyme preparations were used to demonstrate the applicability of the method. The parameter used for the calculation of the enzymatic activity was directly proportional to the enzyme concentration (the dependence was linear in the range of at least 10-fold change in the enzyme concentration). The relative error of the method did not exceed 10%.

Key words: pectin, pectinase, polygalacturonase, polymethylgalacturonase, pectin lyase, pectate lyase, endodepolymerase activity, viscometry

Pectins are important components of many plants [1]. Biodegradation of pectins is catalyzed by a number of enzymes, which differ in their specificities [1-4]. Depolymerization of the pectin backbone formed by residues (usually substituted) of D-galacturonic acid is catalyzed by the following pectinases: 1) polymethylgalacturonase and polygalacturonase (EC 3.2.1.15) hydrolyzing randomly the 1,4-α-D-galactosiduronic linkages in methoxylated or demethoxylated pectins, respectively; 2) pectin lyase (EC 4.2.2.10) depolymerizing pectin by eliminating of the 6-methyl-Δ-4,5-D-galacturonate residues; 3) pectate lyase (EC 4.2.2.2) also exhibiting the elimination mechanism, but towards demethoxylated pectin (pectate). All the enzymes described are characterized by a random (endodepolymerase) type of action on the polymeric substrate. Additionally, the cleavage of the main polymeric chain as well as fragments of its destruction is catalyzed by the exodepolymerases: exopolygalacturonase (EC 3.2.1.67), exopoly-α-galacturonosidase (EC 3.2.1.82), and exopolygalacturonate lyase (EC 4.2.2.9). Lastly, biodegradation of pectins involves such enzymes as pectin esterase (EC 3.1.1.11) and pectin acetylesterase cleaving the methoxyl and acetyl bonds in galacturonans containing the corresponding substituents.

The activity of pectinases of the lyase class can be determined spectrophotometrically by monitoring the formation of the double bond at 232-235 nm [3, 5-7]. The activity of hydrolytic pectinases can be determined either by measuring the reducing sugars or using viscometry [3, 6, 8-19]. Other methods of estimation of the pectinase activity, for example turbidimetric ones [3], are not widely used.

The most popular procedures for determination of the polygalacturonase activity (dinitrosalicylic and Somogyi–Nelson methods) are based on the analysis for reducing sugars using pectate as the substrate [2, 3, 6, 13]. The use of natural pectins (methoxylated galacturonans with different substitution degree) as substrates is limited, because both methods include heating of a sample in a boiling water bath in an alkaline medium. Under these conditions, methoxylated pectins are unstable and thus give a high background level of optical density while assaying for reducing sugars [1, 3].

Viscometric methods for assaying pectinase activity are free from this disadvantage. These methods allow esti-

* To whom correspondence should be addressed.

0006-2979/02/6706-0676$27.00 ©2002 MAIK "Nauka / Interperiodica"

mation of the total endopectinase activity of enzyme preparations reflecting the result of depolymerization of pectin by one, two, or more enzymes of hydrolytic or lyase type of action. However, there is controversial information in the literature concerning viscometric methods, conditions of the assay, and the activity calculation. In some works, the time that was necessary to reduce the viscosity of a pectin solution to a certain extent (for example, 20 or 50%) was used as a measurable parameter for evaluation of the activity [12, 19]. According to other authors, the unit of the activity was defined as the amount of the enzyme that was necessary to achieve a 30 or 50% decrease in the viscosity of a pectin solution after a definite time of the reaction (1, 10, 20, 60, or 120 min) [8, 11, 15-18]. One of the approaches suggested calculating the exponential coefficient β proportional to pectinase activity from the curves of the decrease in the substrate viscosity [14]. Moreover, pectins from different sources and with different degree of methoxylation were used for viscometric studies [8, 12-19]. Thus, literature data on the pectinase viscometry are impossible to compare.

The goal of the present study was to develop a viscometric method for assaying of the total endodepolymerase activity of pectinases, to compare different types of pectins in terms of their applicability in the viscometry, and to compare the endopectinase activity of different enzyme preparations.

MATERIALS AND METHODS

Enzymes. The following commercial enzyme preparations were used: Celloviridine G2X (Belmedprepapaty, Belarus), Bio-Prep 3000 L (Novo Nordisk, Denmark), and NCE L-600 (Dyadic International, USA). Culture filtrates of the fungi *Aspergillus japonicus*, *Penicillium verruculosum*, and *P. canescens* were obtained from the Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences (Pushchino). All preparations were in a liquid state except for the *P. verruculosum* (lyophilized culture liquid).

Substrates. Polygalacturonic acid and citrus and apple pectins were from Sigma (USA); beet pectin was provided by M. V. Gernet (Moscow State University of Food Industry). The content of the methoxyl groups in the citrus pectin was 9%.

Assaying of the polygalacturonase, pectate lyase, and pectin lyase activities. The polygalacturonase activity was assayed using the Somogyi–Nelson method [20, 21]. The procedure was based on the determination of reducing sugars formed during the incubation of 0.5% polygalacturonic acid in the presence of an enzyme preparation (10 min at 50°C and pH 5.0 or 8.0). The pectate lyase and pectin lyase activities were determined by measuring the initial rate of the accumulation of Δ-4,5-unsaturated products of degradation of polygalacturonic acid or citrus pectin, respectively [6]. A spectrophotometric cuvette containing 2.9 ml of the substrate solution (0.24%) in 0.05 M acetate (pH 5.0) or Tris-HCl (pH 8.0) buffer was thermostatted at 40°C; the reaction was started by the addition of 0.1 ml of the enzyme solution, and the kinetics of the accumulation of unsaturated products was recorded at 235 nm. In all cases, one unit of the activity was defined as the amount of the enzyme catalyzing formation of 1 μmol of a product per min of the reaction.

Substrate solution for viscometry. Citrus and apple pectins were dissolved in 0.1 M acetate buffer, pH 5.0, to yield a 1% stock substrate solution, which was stored for 2-3 days at room temperature. To prepare a working pectin solution (0.5%), the stock solution was diluted twice with 0.1 M acetate buffer, pH 5.0. The efflux time of the working pectin solution in the viscometer was 115-120 sec. In the case of beet pectin, a 1.5% working solution was prepared to obtain the same efflux time.

To assay the activity of neutral and mildly alkaline pectinases, the stock and working solutions were prepared in the same way, but using 0.5 M Tris-HCl buffer, pH 8.0. Since pectin is unstable in an alkaline medium, the solutions were prepared on the day of the experiment.

Viscometric assay of the activity of acid pectinases. Viscosity of solutions was measured in a thermostatted Ostwald viscometer (capillary diameter of 0.5 mm, the efflux time for water was 27 sec) using two stopwatches.

To measure the activity of acid pectinases, 5 ml of the working solution (0.5% pectin in 0.1 M acetate buffer, pH 5.0) was placed into the dry and thoroughly washed viscometer thermostatted at 40°C. The solution was preheated at 40°C for 5 min, and then the efflux time T_0 was determined. The measurements were repeated 2-3 times, so that the difference between the values obtained was no more than 1 sec. Then 0.1 ml of a diluted enzyme solution was added by a glass pipette, starting the first stopwatch simultaneously. The solution was mixed carefully (so not to splash), blowing an air through the capillary by a rubber bulb. Then the first measurement of the efflux time of the reaction mixture T_1 was made using the second stopwatch, fixing the time corresponding to the beginning of the first measurement t_1 by the first stopwatch (the moment when the solution passed the upper mark of the viscometer). After the first measurement was made, the reaction mixture was mixed again and the second measurement was made to determine T_2 (the efflux time of the reaction mixture) and t_2 (the time interval between the moment of the addition of the enzyme and the moment when the solution passed the upper mark of the viscometer). To determine T_3 and t_3, T_4 and t_4, the third and forth measurements were made in the same way. In the case the efflux time values T_3 and T_4 differed from the T_0 value less than by 30-40 sec, all measurements were remade, reducing the dilution of the enzyme.

Since the addition of 0.1 ml of the enzyme resulted in some dilution of the solution, a control value of the

efflux time for the original working pectin solution was determined after the addition of 0.1 ml of 0.1 M acetate buffer, pH 5.0. Usually, 3-4 measurements were made so that the difference between the values did not exceed 2 sec. Then the mean value was calculated that was taken as the intrinsic efflux time of the working pectin solution (T_0^{int}). Usually, on the addition of 0.1 ml of the buffer, the ΔT_0 value was 2-3 sec ($\Delta T_0 = T_0 - T_0^{int}$). Such a control was performed once a day for each substrate solution used, and the ΔT_0 value determined was used to calculate the activity of the enzyme investigated.

Calculation of the activity of acid pectinases. For each *i*-th measurement of the efflux time of the pectin solution in the presence of the enzyme (see previous section), a ratio of the relative viscosity of the original pectin solution to the relative viscosity of the same solution containing the enzyme (F_R^i) and the reaction time (t_r^i) were calculated using the following equations:

$$F_R^i = (T_0^{int} - T_B)/(T_i - T_B);$$

$$t_r^i = t_i + T_i/2,$$

where $T_0^{int} = T_0 - \Delta T_0$ and T_B is the efflux time for the buffer. All time values are given in seconds.

Then F_R^i was plotted against t_r^i using linear regression (various computer programs can be used, for example Excel or Origin). Typical dependences F_R^i against t_r^i are presented in Figs. 1b, 3, and 5. The activity of the enzyme is proportional to the slope of the straight line (tanα). The unit of the activity was taken as the activity resulting in a 50% decrease in the relative viscosity of the pectin solution for 5 min of the reaction. Such a decrease in the viscosity corresponded to the change in the F_R^i value by 1 for 300 sec. Thus, one unit of pectinase activity corresponded to the slope of 0.0033 of the straight line $\{F_R^i; t_r^i\}$. Since we added into the viscometer 0.1 ml of the enzyme solution, one unit of the pectinase activity was provided by the enzyme solution of 10 U/ml.

Depending on the slope of the straight line $\{F_R^i; t_r^i\}$, the equation for the calculation of the activity is the following:

$$A\ (\text{U/ml}) = 3000 \cdot \tan\alpha \cdot R,$$

where R is the dilution of the enzyme before its addition to the substrate solution in the viscometer.

Viscometric assay of the activity of neutral (mildly alkaline) pectinases at pH 8.0. The experimental procedure for assay of the activity at pH 8.0 was similar to that described for pH 5.0 (see above). However, since pectin was not quite stable at pH 8.0 and enhanced temperature (40°C), it was gradually subjected to spontaneous degradation by the β-elimination mechanism. So, the control and the calculation of the activity were more complex (see "Results" for details).

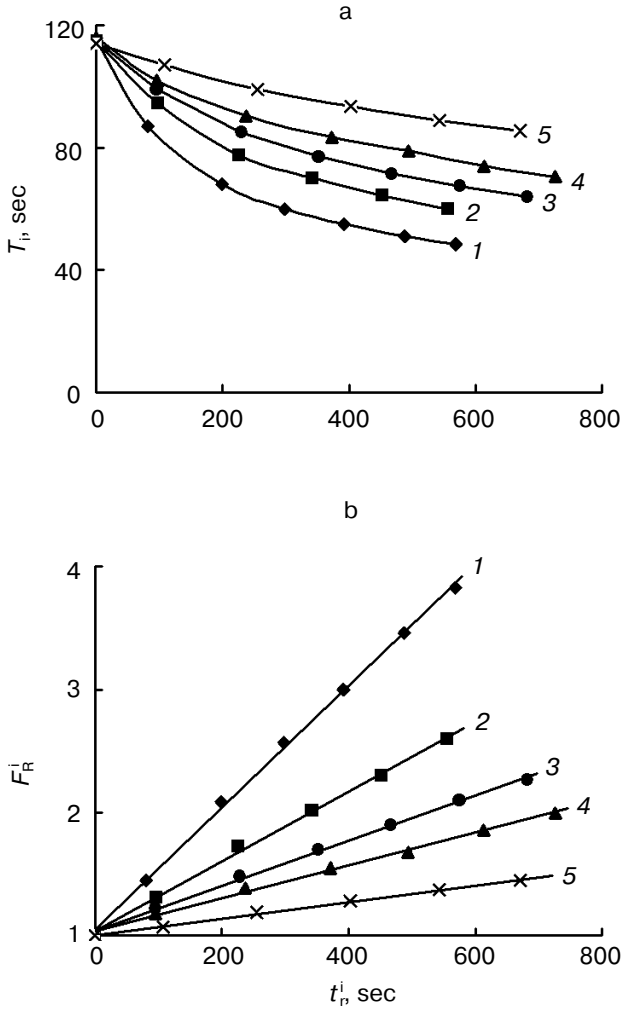

Fig. 1. a) Changes in the efflux time of a citrus pectin solution in the presence of different dilutions (R = 10, 20, 30, 40, and 100) of *A. japonicus* sample (curves *1-5*, respectively); b) linearization of the data in the coordinates employed for the calculation of the endopectinase activity: $y = 0.0049x + 1.0526$ (*1*), $0.0028x + 1.0336$ (*2*), $0.0018x + 1.034$ (*3*), $0.0013x + 1.0373$ (*4*), $0.0007x + 1.0043$ (*5*).

RESULTS

The method for estimation of the total endopectinase activity uses similar procedure as the standard viscometric method for assay of the hemicellulase activity [22].

Viscometric assay of acid pectinase activity (pH 5.0). Figure 1a presents dependences of the efflux time of the citrus pectin solution on time of the enzymatic reaction in the presence of different dilutions of the enzyme preparation of *A. japonicus*. Figure 1b presents the same data as a plot F_R^i against t_r^i. Figure 2 shows the dependence of the slopes of the straight lines $\{F_R^i; t_r^i\}$ on concentration of the enzyme preparation (*1*). As seen from the

figures, the data on the change in the viscosity of the pectin solution (Fig. 1a) can be linearized in the suggested coordinates reasonably well (Fig. 1b), and the rate of the change in the ratio of the relative viscosities F_R^i (i.e., tanα) is proportional to the enzyme concentration in a wide range (Fig. 2). In all cases of the linear regression, the correlation coefficient was close to 1 (0.997-0.999), this suggesting the possibility of the use of the described method for assaying of the pectinase activity in practice. In a perfect case, the absolute term of the equation of a linear regression (i.e., the point where the straight line cuts the ordinate) must be 1. In the equations of the linear regression obtained using the data presented in Fig. 1b this value was close to 1 (it varied from 1.004 to 1.053), this indicating the adequacy of the method.

Figure 3 demonstrates the changes in the pectin solution viscosity in the presence of a number of other enzyme preparations at pH 5.0. It should be noted that since the preparations were diluted before the experiment to a different extent, slopes of the straight lines in Fig. 3 do not reflect the ratio of endopectinase activities of the original preparations. As seen from the figure, the plot F_R^i versus t_r^i yielded good linear dependences for all samples. The data for Celloviridine (plot *3*) were linearized worse in the given coordinates, and the same character of the dependence was observed for different concentrations (dilutions) of this preparation. For all enzyme preparations, the rate of the change in F_R^i depended linearly on the enzyme concentration (data for Celloviridine are presented in Fig. 2, data for other enzymes are not shown). The results of the viscometric assay of the endopectinase activity of different preparations are summarized in Table 1.

Comparison between different types of pectins. Three different types of pectin (citrus, apple, and beet) were compared in terms of their applicability in the viscometric assay of the pectinase activity using three enzyme preparations: Celloviridine G2X, *A. japonicus*, and NCE L-600 (at pH 5.0). Kinetic curves reflecting the changes in the efflux time of apple and beet pectin solutions in the presence of enzymes were similar to those obtained with citrus pectin (see above), and the data were linearized reasonably well in the suggested coordinates. The activity values of Celloviridine G2X as well as preparations of *A. japonicus* and NCE L-600 determined using citrus (0.5%), apple (0.5%), and beet (1.5%) pectins were 499, 158, and 30; 478, 141, and 45; and 375, 177, and 6 U/ml, respectively. The data obtained with apple and citrus pectins were in good agreement, while the activity values obtained with beet pectin significantly differed from those obtained using apple and citrus pectins as substrates. Citrus pectin was chosen for subsequent studies as a well-characterized and accessible substrate, also being the most convenient one (it provided the most stable efflux time value of the solution).

Viscometric assay of the activity of neutral and mildly alkaline pectinases (pH 8.0). As mentioned above, while measuring the pectinase activity in an alkaline medium and enhanced temperature using methoxylated pectins as substrates, special attention should be paid to control experiments, because under such conditions pectins can be subjected to spontaneous degradation by the β-elimination mechanism [1, 3].

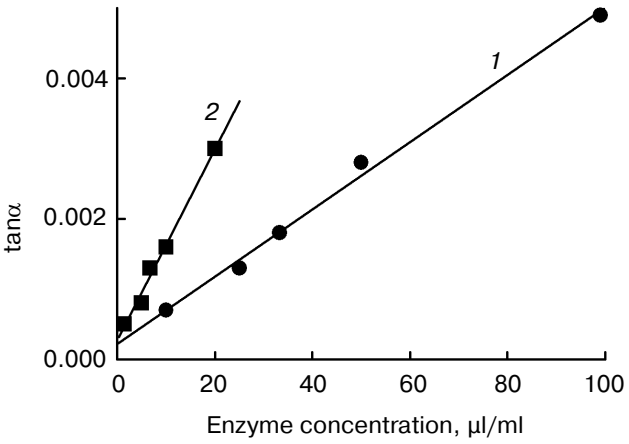

Fig. 2. Effect of the enzyme concentration on the rate of the change in the ratio of the relative viscosities (tanα) for enzyme preparation from *A. japonicus* (*1*, $y = 0.00005x + 0.00022$, $R^2 = 0.99518$) and Celloviridine G2X (*2*, $y = 0.00014x + 0.00026$, $R^2 = 0.98943$).

Figure 4 presents the dependence of the efflux time of the citrus pectin solution on time of incubation at 40°C

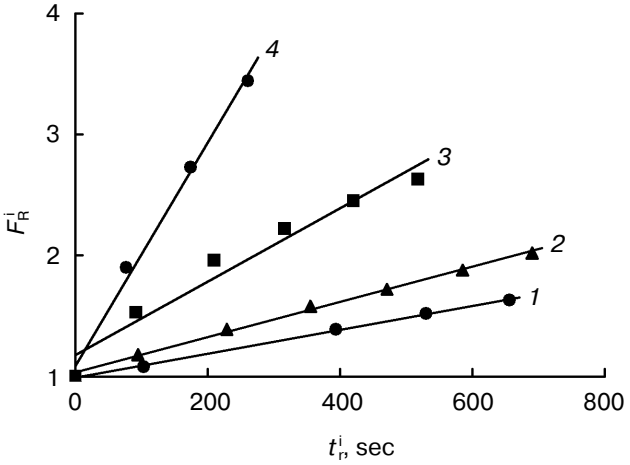

Fig. 3. Data on changes in the viscosity of the citrus pectin solution in the presence of different enzyme preparations at pH 5.0 in the coordinates used for the calculation of the endopectinase activity: *1*) NCE L-600 ($R = 10$, $y = 0.001x + 0.9929$); *2*) *P. canescens* ($R = 10$, $y = 0.0015x + 1.0344$); *3*) Celloviridine G2X ($R = 50$, $y = 0.003x + 1.1779$); *4*) *P. verruculosum* (20 g/liter) ($y = 0.0092x + 1.0855$).

Table 1. Pectinase activity of different enzyme preparations

Preparation	Activity towards citrus pectin (viscometry), U/ml		Polygalacturonase, μmol/min per ml		Pectate lyase, μmol/min per ml		Pectin lyase, μmol/min per ml	
	pH 5.0	pH 8.0	pH 5.0	pH 8.0	pH 5.0	pH 8.0	pH 5.0	pH 8.0
A. japonicus	158 ± 9	12 ± 1	140 ± 7	< 1	0	0	1.6 ± 0.1	0
P. canescens	45 ± 2	3.9 ± 0.2	43 ± 1	< 1	0.8 ± 0.1	< 0.1	0.4 ± 0.1	< 0.1
*P. verruculosum**	1450 ± 50	120 ± 8	1248 ± 90	0	< 0.4	< 0.4	13 ± 1	< 0.4
Celloviridine G2X	499 ± 51	0	602 ± 45	4 ± 1	< 0.2	< 0.2	2.0 ± 0.1	0
Bio-Prep 3000 L	7.1 ± 0.2	630 ± 60	13 ± 1	289 ± 16	< 0.1	266 ± 8	3.1 ± 0.1	168 ± 2
NCE L-600	30 ± 2	795 ± 54	40 ± 2	18 ± 1	1.3 ± 0.1	16 ± 1	< 0.2	69 ± 5

* The activity values are calculated per g of the lyophilized preparation.

and pH 8.0 in the absence of the enzyme. As seen from the data, some decrease in the viscosity of the substrate solution occurred due to a spontaneous (nonenzymatic) degradation of the polymer during the incubation time usually employed to determine the enzymatic activity. Therefore, the procedure of the viscometric assay and the calculation of the endodepolymerase activity of pectinases at pH 8.0 were modified in comparison with the method developed for pH 5.0. At pH 8.0, after preheating of the substrate solution in the viscometer for 5 min, the efflux time T_0 of the original solution was determined once (not 2-3 times as described for pH 5.0), and then no more than 3-4 measurements of T_i were made so as to minimize the error connected with the nonenzymatic degradation of the polymer. The parameters F_R^i and t_r^i were calculated using the following equations:

$$F_R^i = (T_0 - \Delta T_t - T_B)/(T_i - T_B)$$

$$t_r^i = t_i + T_i/2 ,$$

where ΔT_t was estimated using Table 2 in accordance to t_r^i. The parameter ΔT_t is the correction reflecting the decrease in the efflux time of the working pectin solution due to the following reasons: a) slight dilution of the reaction mixture caused by the addition of 0.1 ml of the enzyme (this value is accepted to be 3 sec), and b) spontaneous degradation of the substrate. The data for (b) are presented in Fig. 4.

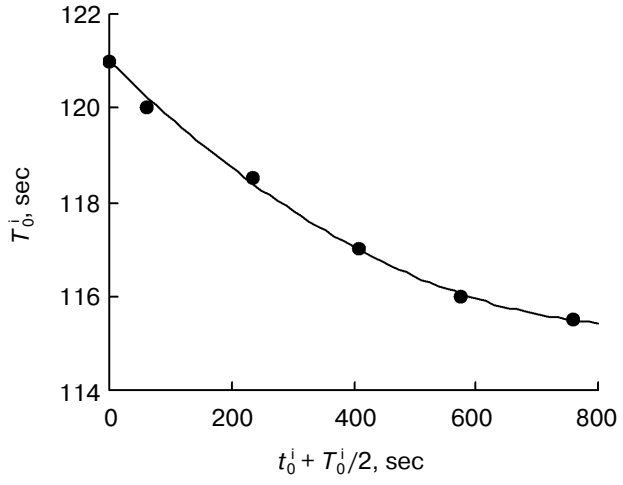

Fig. 4. Change in the efflux time of citrus pectin solution due to spontaneous degradation at 40°C and pH 8.0 (measurements were started after preheating of the solution in the viscometer for 5 min).

In Fig. 5, the data on the rate of the change in the viscosity of the pectin solution in the presence of two enzyme preparations at pH 8.0 are presented in the $\{F_R^i; t_r^i\}$ coordinates. In the case of NCE L-600, experimental points were approximated by the linear regression reasonably well. For the preparation Bio-Prep 3000 L, the dependence was nonlinear (curve *1* shown by points); the linear approximation of the data yielded a straight line (*2*) that cut the ordinate at a point significantly different from 1. In the case of NCE L-600 (plots *3* and *4*), the straight line *3* was calculated making the correction for the spontaneous degradation of the substrate at pH 8.0 (ΔT_t), while the straight line *4* was created using the method of the activity calculation developed for pH 5.0 (incorrect calculation method was done to illustrate the importance of the correction ΔT_t). As seen from the plots *3* and *4*, the calculation of the activity without making the correction for the spontaneous degradation of pectin resulted in the enhanced activity values.

Table 2. Corrections ΔT_t reflecting the decrease in the efflux time of 0.5% citrus pectin solution at 40°C and pH 8.0 depending on the reaction time t_r^i

t_r^i, sec	0–50	50–100	100–200	200–350	350–600
ΔT_t, sec	3	4	5	6	7

The data on the endodepolymerase activity of pectinases from different enzyme preparations at pH 8.0 are presented in Table 1.

DISCUSSION

In the present work, one unit of the endodepolymerase activity was defined as the activity resulting in 50% decrease of the relative viscosity of 0.5% citrus pectin solution for 5 min of the enzymatic reaction at 40°C and the appropriate pH (5.0 or 8.0). It should be noted that the activity was calculated using several experimental values of the efflux time of the substrate solution determined within 1-10 min of the reaction. The data of several measurements were used to plot a straight line using linear regression, this increasing the accuracy of the activity calculation. The time interval chosen for the calculation of the activity (5 min) corresponded to the typical mean time needed for the activity measurements. For five preparations investigated, the data on the decrease in the substrate viscosity during the enzymatic reaction were linearized in the described coordinates reasonably well, and the parameter used for calculation of the activity (tanα) was directly proportional to the enzyme concentration in the range of at least 10-fold changes. The correlation coefficient was close to 1 (typical value was 0.99). In only one case (for Bio-Prep 3000 L), a nonlinear dependence F_R^i versus t_r^i was observed. Presumably, such a nonlinear dependence was not a disadvantage of the viscometric method, but was due to a characteristic feature of the catalytic mechanism of the enzyme (the nonlinear character of kinetic curves was also observed for pectate lyase Bio-Prep 3000 L in the case of spectrophotometric detection of the reaction products at 235 nm; a detailed study of the catalytic mechanism of this enzyme was outside of the purpose of the present study).

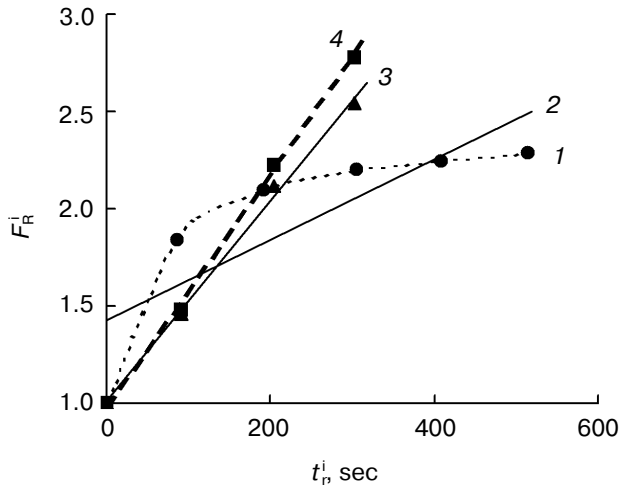

Fig. 5. Data on changes in the viscosity of citrus pectin solution in the presence of different enzyme preparations at pH 8.0 in the coordinates used for calculation of the endopectinase activity: *1*, *2*) Bio-Prep 3000 L, $R = 100$; experimental points (*1*) and their approximation by linear regression $y = 0.0021x + 1.4236$ (*2*); *3*, *4*) NCE L-600, $R = 50$; the data were calculated making the corrections for spontaneous degradation of pectin, $y = 0.0052x + 1.0061$ (*3*), or without making the corrections, $y = 0.006x + 0.9814$ (*4*).

Table 1 presents the pectinase activity of the studied preparations. The activity was determined at pH 5.0 and 8.0 using different methods: viscometric method, assay for reducing sugars (polygalacturonase), and spectrophotometric detection at 235 nm (pectate lyase and pectin lyase). As seen from the results presented, the viscometric method allows measuring the activity of the enzymes of different classes, both hydrolytic (polygalacturonase) and lyase type of action. In the case of the first four preparations presented in Table 1, the activity determined viscometrically reflected mainly the depolymerizing action of hydrolytic pectinases, because these preparations possessed a high polygalacturonase activity determined by measuring the reducing sugars and an extremely low (or zero) lyase activity. In the case of the preparations Bio-Prep 3000 L and NCE L-600, the main contribution into the activity determined viscometrically was made by the lyase type enzymes. It should be noted that the weight content of the methoxyl groups in citrus pectin constituted 9%, this corresponding to ~70% esterification degree (percentage of the esterified carboxyl groups with respect to the total number of the carboxyl groups). The substrate used is a highly methoxylated pectin [1]. Thus, the viscometric method suggested for the assay of the endopectinase activity can also reflect the depolymerizing action of polymethylgalacturonases, although the best substrates for these enzymes are pectins with esterification degree more than 90% [18].

Generally, measurement error for the determination of the endopectinase activity by the viscometric method

was 3-7% (maximally, it does not exceed 10%), this being comparable with the accuracy of spectrophotometric methods of the pectinase activity assay (Table 1). A certain advantage of the viscometric method is the fact that impurities that are present in enzyme preparations do not affect the accuracy of the measurements, but they may hinder the assay for reducing sugars or Δ-4,5-unsaturated products of the substrate degradation while measuring the polygalacturonase or lyase activities.

REFERENCES

1. Voragen, A. G. J., Pilnik, W., Thibault, J.-F., Axelos, M. A. V., and Renard, M. G. C. (1995) in *Food Polysaccharides and Their Applications* (Stephen, A. M., ed.) Marcel Dekker, Inc., New York, pp. 287-339.
2. Kertesz, Z. I. (1955) *Meth. Enzymol.*, **1**, 158-166.
3. Rexova-Benkova, L., and Markovic, O. (1976) *Adv. Carbohydr. Chem. Biochem.*, **33**, 323-385.
4. Blanco, P., Sieiro, C., and Villa, T. G. (1999) *FEMS Microbiol. Lett.*, **175**, 1-9.
5. Moran, S., Nasuno, S., and Starr, M. P. (1968) *Arch. Biochem. Biophys.*, **123**, 298-306.
6. Collmer, A., Ried, J. L., and Mount, M. S. (1988) *Meth. Enzymol.*, **161**, 329-335.
7. Konno, H. (1988) *Meth. Enzymol.*, **161**, 381-385.
8. Tuttobello, R., and Mill, P. J. (1961) *Biochem. J.*, **79**, 51-64.
9. Bateman, D. F. (1963) *Phytopathology*, **53**, 1178-1184.
10. Voragen, A. G. J., Rombouts, F. M., Hooydonk, M. J., and Pilnik, W. (1971) *Lebensm. Wiss. Technol.*, **4**, 7-11.
11. Cleveland, T. E., and McCornick, S. P. (1987) *Phytopathology*, **77**, 1498-1503.
12. Tagawa, K., and Kaji, A. (1988) *Meth. Enzymol.*, **161**, 361-365.
13. Schejter, A., and Marcus, L. (1988) *Meth. Enzymol.*, **161**, 366-373.
14. Lopez, P., Fuente, J. L., and Burgos, J. (1994) *Analyt. Biochem.*, **220**, 346-350.
15. Maiorano, A. E., Schmidell, W., and Ogaki, Y. (1995) *World J. Microbiol. Biotechnol.*, **11**, 355-356.
16. Acuna-Arguelles, M. E., Gutierrez-Rojas, M., Viniegra-Gonzalez, G., and Favela-Torres, E. (1995) *Appl. Microbiol. Biotechnol.*, **43**, 808-814.
17. Galiotou-Panayotou, M., Kapantai, M., and Kalantzi, O. (1997) *Appl. Microbiol. Biotechnol.*, **47**, 425-429.
18. Pashova, S., Slokoska, L., Krumova, E., and Angelova, M. (1999) *Enzyme Microb. Technol.*, **24**, 535-540.
19. Astapovich, N. I., and Ryabaya, N. E. (2000) *Prikl. Biokhim. Mikrobiol.*, **36**, 160-163.
20. Nelson, N. (1944) *J. Biol. Chem.*, **153**, 375-379.
21. Somogyi, M. (1952) *J. Biol. Chem.*, 1952, **195**, 19-23.
22. *Food Chemicals Codex* (1996) 4th ed., National Academy Press, Washington, D. C., pp. 799-800.