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MARTA MITEK
BOHDAN DRZAZGA

INTERRELATION BETWEEN THE EFFECT OF ENZYMATIC CLARIFICATION OF APPLE JUICES AND THE AMOUNT AND QUALITY OF POLYPHENOLS. I. INHIBITION OF PECTINOLYTIC ENZYMES BY POLYPHENOLS. STUDIES OF MODEL SYSTEMS

Department of Fermentation and Fruit and Vegetable Technology, Warsaw Agriculture University

Key words: polyphenols, inhibition, polygalacturonase (PG), pectinesterase (PE), pectinolysis pectinolytic preparations, apple juice

The effect of 15 polyphenolic compounds on the activity of polygalacturonases and poctinesterase contained in selected pectinolytic preparations (Pectopol PT, Pectinase and Panzym Super) was studied. The tests performed in model systems demonstrated that all the investigated polyphenolic compounds with the exception of chlorogenic, caffeic and protocatechuic acids inhibit the activity of both polygalacturonases and pectinesterase. The strongest enzyme inhibitors among the considered polyphenols were dl-catechol, apigenin, koempferol, quercitrin, quercetin as well as m- and p-coumaric acids. Ferulic acid inhibits pectinesterase activity fairly substantially but has no inhibitory effect on polygalacturonases activity.

Despite being known for years, enzymatic processing of apple juice aimed at pectins decomposition often causes serious problems, with the resultant juice containing insufficiently degraded pectins. One of the possible reasons for this may be the presence in the juice of polyphenols which are said to be capable of inhibiting reactions with many enzymes, including also pectinolytic ones.

The studies of pectinolytic enzymes inhibition by polyphenols (e.g. Goldstein [5], Pollard [11], Hathway [6], Lech [8]) show that the inhibiting compounds are mainly high-molecular tannins, oxidized leucoanthocyanidins and catechols. According to Williams [15] one may add to this group also certain derivatives of cinnamic acids as well as coumaric acids. Opinions as to the inhibitory properties of chlorogenic acids and of other compounds included in the group of phenyl-propenic acids (namely ferulic and caffeic acids) are divided, a situation which may be due to different conditions of the various studies, mainly the kind of pectin substrate and the applied pectinolytic enzymes.

The objective of Part I of this research was to determine which of the polyphenols occurring in apple juice inhibit polygalacturonases and pec-

tinesterase contained in selected pectinolytic preparations, and to assess the intensity of this inhibition.

MATERIALS AND METHODS

The substrate used in the experiments was an apple pectin preparation (obtained from the Fruit and Vegetable Processing Plant in Jasło) with the following characteristic: dry substance 94.2%, purity 66.1%, esterification degree 67.0%.

The enzymes sources were three pectinolytic preparations: Pectopol PT manufactured by the Jasło Fruit and Vegetable Processing Plant; Panzym Super from C. H. Boehringer und Sohn (ERG), and Pectinase obtained from Koch Light Lab. Ltd. (Great Britain). Pectopol PT and Panzym super are multienzymatic preparations containing, in addition to polygalacturonases and pectinesterase, also amylases and cellulases. Optimum pH for both preaparations is in the range 3.5-5.0, while the optimum temperature range is 20-25°C. Pectinase, on the other hand is a purified polygalacturonases preparation for laboratory studies.

The polyphenol compounds to be studied were chosen from among those occurring in apples and in apple musts. A total of 15 such compounds were selected: shikimic and ferulic acids (manufactured by Fluka AG, Buchs SG in Switzerland), protocatechuic acid (Aldrich-Europe Janssen Pharmaceutica, Belgium), o-coumaric acid (Schuchardt München, FRG), m-coumaric acid, apigenin, quercitrin, kalmpferol, dl-catechol (all from ICN Pharmaceutical, Inc., U.S.A.), p-coumaric acid and chlorogenic acid (from Koch Light Lab. Ltd., Great Britain), quercetin (Lachema, Czechoslovakia), rutin (Merck, FRG) and tannic acid (Loba Chemie Wien-Fischamend, Austria).

The inhibition of polygalacturonases by polyphenols was determined by measuring decreases of viscosity of the model pectin solutions. The conditions of experiments were as follows: 0,5% solution of pectin preparation in Mc Ilvaine buffer of pH 3.5, Pectopol PT concentration — 0.05%, Pectinase concentration — 0.012%, temperature — 30°C, reaction time — 0-180 min. In the case of caffeic acid, quercetin and rutin, 10 cm^3 of 96% ethyl alcohol were added to every 100 cm^3 of the model solution. In the first series tests, polyphenols were added to the pectin solution in amounts similar to those found in apple juice, in the form of water or alcohol solution (caffeic acid, quercetin, rutin); in test series II and III the polyphenols were combined with the enzymatic preparation solution for 30 min and only then added to the pectin preparation solution.

The viscosity of model solutions was measured in a Höppler viscosimeter at 15 min intervals during 3 h of reaction. The percent drop of viscosity was calculated as the so called hydrolysis number (A) for the various ball drop times (V) after the successive reaction times (T) from the formula

$$A\% = \frac{V_o - V_{1,2,3,\ldots\ldots,12}}{V_o - V_k} \cdot 100,$$

where A is the hydrolysis number (%), V_0 — initial viscosity of the solution (without the enzymatic preparation addition) as ball drop time (sec), $V_{1,2,3,.....12}$ — viscosity of the mixture with the enzymatic preparation after a specific time of enzyme activity (15-180 min) as ball drop time (sec), V_k — final viscosity of the mixture after 24 h of enzymatic preparation activity (reference test) as ball drop time (sec).

The degree of inhibition was calculated for the individual polyphenols basing on the reaction time at which the hydrolysis number in the control sample (without an inhibitor) ranged from 50 to 60%. The formula used was

$$\text{Inhibition } \% = \frac{A_1 - A_2}{A_1} \cdot 100,$$

where A_1 — hydrolysis number (50-60%) after a specific reaction time (control), and A_2 — hydrolysis number after the same reaction time in samples with an inhibitor.

The effect of polyphenols on pectinesterase activity was studied using the Panzym Super preparation in the following experiment conditions: 1% pectin preparation solution in 0.1 M NaCl solution, Panzym Super concentration — 0.15%, temperature 30°C. The degree of pectinesterase inhibition was calculated from the formula used in the case of polygalacturonases, but with the hydrolysis number (A) replaced by pectinesterase activity determined by potentiometric titration [12].

The obtained results were interpreted statistically by means of variance analysis using the Fischer-Snedecor and Dunnettests. Linear correlation coefficients were calculated for results of test series III and described with regression equations of type $y = ax + b$ and with exponential function equations of type $y = ax^b$.

RESULTS AND DISCUSSION

SERIES I OF EXPERIMENTS

The effect of 15 polyphenol compounds on the activity of polygalacturonases contained in Pectopol PT and Pectinase and of pectinestarese in the Panzym Super preparation is illustrated in Table 1; the statistical assessment of the results obtained in both series of experiments is given in Table 2.

As can be seen, the inhibitory effect of the studied polyphenols on polygalacturonases contained in Pektopol PT is slight, ranging from 0% for ferulic, caffeic and chlorogenic acids to 15% for rutin. According to statistical analysis, the effect of nine of the investigated 15 polyphenols on polygalacturonases in Pektopol PT may be regarded as statistically significant. These compounds are: o-coumaric, shikimic, m-coumaric and tannic acids, quercetin, quercitrin, kaempferol, apigenin and rutin.

Table 1. Inhibition of polygalacturonases and pectinesterase by polyphenols (series I of experiments)

Polyphenol compound	Concentration in model solution (mg/100 cm³)	Inhibition of		
		polygalacturonases from		pectinesterase from Panzym Super
		Pektopol PT	Pectinase	
shikimic acid	4.0	6.5	15.0	5.0
protocatechuic acid	20.0	4.0	20.5	3.5
o-coumaric acid	20.0	5.0	19.5	16.0
m-coumaric acid	0.5	7.0	21.0	17.5
p-coumaric acid	2.5	2.0	16.0	12.0
ferulic acid	20.0	0.0	13.5	28.0
caffeic acid	20.0	0.0	5.0	6.5
chlorogenic acid	30.0	0.0	14.5	9.0
apigenin	0.1	12.5	18.5	5.0
quercetin	10.0	9.0	42.5	25.5
quercitrin	3.0	8.5	14.5	9.0
rutin	20.0	15.0	53.5	18.5
kempherol	0.5	11.0	21.0	10.0
dl-catechol	50.0	2.0	39.5	27.0
tannic acid	30.0	7.5	47.0	17.0

Table 2. Results of variance analysis

Source of variability	Degrees of freedom	Polygalacturonases from								$F_{0.05}$
		Pektopol PT		Pectinase						
		variance	F_{emp}	Series I		Series II		Pectinesterase from Panzym Super		
				variance	F_{emp}	variance	F_{emp}	variance	F_{emp}	
Kind of polyphenol	15	31.4	9.5	174.8	52.0	78.6	21.0	53.0	945.2	
Error	32	3.3		3.4		3.7		0.06		2.017

The considered polyphenols turned out to be stronger inhibitors of polygalacturonases contained in Pectinase. The degree of inhibition in this case ranged from 5% for caffeic acid to about 54% for rutin, and statistical analysis confirmed the significance of the inhibitory effect of 14 of the 15 compounds (the sole exception being caffeic acid). Worth noting are compounds which did not inhibit polygalacturonases in Pectopol PT but inhibited these enzymes in the Pectinase preparation. These include ferulic and chlorogenic acids (0% inhibition for Pectopol PT and 13,5% inhibition, respectively, for Pectinase), protocatechuic acid (4,0 and 20,5% inhibition for Pectopol PT and Pectinase, respectively), p-coumaric acid (2,0 and 16,0%) and dl-catechol (2,0% and 30,5%). These differences in degree of inhibition are probably due to different degrees of

enzymatic preparation purity, with the unrefined enzymes being more resistant to unfavourable environmental conditions, including various kinds of inhibitors [1, 8].

The obtained results are in accord with data published by Ishii [7] and Pollard [11] who studied the effect of leucoanthocynidines isolated from pears on the activity of polygalacturonases of various origin and found that at 0,55% concentration of inhibitor, pure polygalacturonases became completely inactive, while commercial preparations of these enzymes remained immune to this effect. Also important is the origin of the enzyme which significantly determines its properties, including sensitivity to various inhibitors. Although both Pectopol PT and Pectinase are obtained from *Aspergillus niger mycelium*, the former is characterized mainly by endo-polygalacturonase activity, while the latter is a purified form of exo-polygalacturonase.

The second enzyme determining the correct course of enzymatic clarification of media is pectinesterase, which acts by preparing the pectin substrate for polygalacturonases through its partial de-estrification. This is a very important process since apple pectins exhibit a relatively high degree of estrification, unfavourable for the operation of these enzymes. On the other hand de-estrification of the pectin substrate is unwellcome in view of the liberation of methanol and of the possibility of precipitation of pectic acids and their salts which leads to opacity of the finished product. Given this, a partial inhibition of pectinesterase would be desirable, especially if it were to take place after a preliminary de-estrification of pectins.

The results obtained in this research (Table 1) indicate that all of the studied polyphenols inhibit the activity of pectinesterase. However, compared to the effect of the same amounts of polyphenols on the activity of polygalacturonases from Pectopol PT and Pectinase, the inhibition of pectinesterase is much weaker. The most effective inhibitors of this enzyme are ferulic acid, dl-catechol, quercitrin, rutin, tannic acid and o-, m- and p-coumaric acids. Particularly noteworthy among these compounds is ferulic acid, which did not inhibit polygalacturonases at all but lowered the activity of pectinesterase by as much as ca 30%. The available literature lacks data on the selective effect of this compound on pectinesterase activity, although it was found to be an inhibitor of o-diphenol oxidase [14]. From the point of view of enzymatic pectinolysis of apple juices or pulps, a partial inhibition of pectinesterase by means of the selectively and specifically acting ferulic acid might be useful and advisable, the more so since, acting simultaneously on o-diphenol oxidase, it prevents oxidation (e.g. of catechols) thereby adversaly affecting the activity of polygalacturonases.

Summing up the results of the first series of experiments, we may say that except for caffeic acid which does not act as an inhibitor and rutin which is most effective as an inhibitor, the studied polyphenols acted variously, their inhibitory effect being strongest in the case of polygalacturonases contained in the Pectinase preparation.

SERIES II OF EXPERIMENTS

This series of tests was aimed at determining the effect of polyphenols on polygalacturonases activity in conditions of direct enzyme-inhibitor contact. The results of these experiments are collected in Table 3.

Table 3. Inhibition by polyphenols of polygalacturonases contained in the preparation Pectinase (series II of experiments)

Polyphenol compound	Concentration in Pectinase pectinolytic preparation solution (mg/100 cm^3)	Concentration in the model solution (mg/100 cm^3)	Inhibition (%)
shikimic acid	4.0	0.08	12.0
protocatechuic acid	20.0	0.4	7.0
o-coumaric acid	20.0	0.4	18.0
m-coumaric acid	0.5	0.01	18.5
p-coumaric acid	2.5	0.05	14.0
ferulic acid	20.0	0.4	12.0
caffeic acid	20.0	0.4	9.0
chlorogenic acid	30.0	0.6	14.0
apigenin	0.1	0.002	10.0
quercetin	10.0	0.2	26.0
quercitrin	3.0	0.06	12.0
rutin	20.0	0.4	12.0
kempherol	0.5	0.01	15.0
dl-catechol	50.0	1.0	46.5
tannic acid	30.0	0.6	14.5

As we can see, despite the 50 times lower polyphenols concentration in the model solutions, their inhibition was in most cases similar to that observed in the first series of experiments ranging from 7.0% (protocatechuic acid) to about 47% (dl-catechol). Only rutin and tannic acid produced different effects in both cases. In series I their effect was pronounced (53.5 and 47.0% inhibition, respectively) while in series II it was much weaker (12.0 and 14.5% inhibition, respectively).

It may be surmised that such high values of inhibition for nearly all the polyphenols, and this despite their considerably reduced concentration, is due to the formation of enzyme-inhibitor complexes which may cause a partial destruction of the secondary structure of enzymatic protein, and hence also a drop in enzymatic activity.

The ability of low-molecular polyphenols to combine with proteins is reported e.g. by Singleton [13] who claims that the bonds acting a decisive role in these combinations are hydrogen bonds. Hence, the stability of the emerging complexes is greater when the phenol compound involved is of a more acid character. Polyphenols-enzymes complexes are also discussed by many authors studying the effects of tannins on various enzymes [3, 6, 9, 10].

Tannins are recognized as compounds capable of combining with proteins, including also enzymatic ones, and it depends on their character and on environmental conditions whether the resultant complexes inactivate the enzymes or merely cause a temporary reduction of their activity. Studies of Olson and Stanley [10] demonstrate that the tannin-enzyme complex does not cause the loss of enzyme activity but only its temporary inhibition. Hathway [6] arrived at similar conclusions when he found that the tannin-enzyme complex that is formed undergoes gradual dissociation in the pectin solution, liberating the enzyme in the process which in turn leads to a slight increase of the enzyme's activity.

Studies of tannic acid confirm the above hyphotesis about the transitory character of tannin-protein combinations. Without ruling out the possibility of a combination of tannin with the polygalacturonase enzyme into a partially inactive complex of the inhibitor-enzyme type, it may be surmised that the higher level of inhibition obtained in the first series of experiments may be a result of possible combinations of tannin and pectins. This possibility is discussed, among others, by Goldstein [5] and Hathway [6] who are of the opinion that the weakening of polygalacturonases activity by tannins present in the medium is not only due to the emerging tannins-enzymatic proteins combinations which are of temporary character. The main reason for the enzyme activity drop is the formation of tannin-pectins combinations which block the substrate, thereby making it unavailable to the enzyme.

It may be generally assumed that the mechanism of the inhibitory effect of tannins and other polyphenol compounds on pectinolytic enzymes consists on the one hand in the formation of polyphenols-enzyme combinations and, on the other, in blocking the substrate by means of polyphenols-pectins complexes. Thus, the ultimate effect of the inhibitors depend not only on the kind and amound of the inhibitor itself but also on the kind of the substrate.

SERIES III OF EXPERIMENTS

In this series we attempted to determine the dependence between increasing concentrations of polyphenolic compounds and their inhibitory effect on polygalacturonases contained in the Pectinase preparation. Statistical calculations revealed that increasing concentrations of all the considered polyphenols bring about significant drops of polygalacturonases activity; the greatest changes in inhibition degree took place at low concentrations of inhibitors (up to about 10 mg per 100 cm^3 of model solution). There was fairly substantial defferentiation in the degree of inhibition depending on the applied inhibitor (Tab. 4).

Comparing the degree of inhibition brought about by the various polyphenol compounds (in concentrations of up to 10 mg/100 cm^3), we can see that most active inhibitor of pectinolytic enzymes is kaempferol (ca. 40% reduction of polygalacturonases activity) followed by quercetin and quercitrin) both causing a ca. 26% drop of activity).

Table 4. Dependence of the inhibition of polygalacturonases contained in the Pectinase preparation on the concentration of polyphenol inhibitor

Inhibitor	Inhibitor concentration (mg/100 cm^3)								
	1	5	10	20	40	80	160	320	640
	Inhibition (%)								
shikimic acid	5.0	12.5	15.0	18.0	22.0	25.0	24.0	25.0	—
protocatechuic acid	0.0	1.5	2.5	7.5	10.0	20.6	24.9	26.7	20.7
o-coumaric acid	1.5	4.5	9.0	17.7	19.2	24.3	28.3	40.0	42.1
m-coumaric acid	13.5	18.5	19.0	25.0	26.0	31.0	30.0	31.0	—
p-coumaric acid	13.5	18.0	19.2	22.0	20.0	20.4	19.2	—	—
ferulic acid	2.0	4.5	7.2	11.8	13.6	12.8	20.9	19.5	21.1
chlorogenic acid	1.0	3.0	5.0	8.4	15.2	19.7	24.8	27.2	35.1
caffeic acid	0.0	2.5	5.0	9.1	12.8	19.5	17.0	14.8	21.1
apigenin	10.0	20.0	20.3	18.7	19.2	21.0	21.5	—	—
quercetin	16.0	17.5	26.2	26.2	27.7	43.8	50.6	61.4	74.4
quercitrin	10.0	12.0	26.6	34.4	30.0	34.6	35.0	—	—
rutin	2.0	6.5	11.0	12.0	17.5	29.3	23.3	51.4	72.1
kempherol	20.3	25.6	38.3	33.5	35.0	—	—	—	—
dl-catechol	5.0	10.0	17.5	26.2	38.2	52.7	64.3	71.4	79.6
tannic acid	0.0	0.0	10.0	14.7	14.7	18.7	20.0	35.8	48.5

In assessing the role of individual phenolic compounds in enzymatic processing of apple juices one must take into consideration their actual concentration in the material subjected to pectinolysis. Studies showed that compounds included in the flavonols group, i.e. quercetin and kaempferol are confined mainly to apple skin, and therefore do not pass in large amount to the juice [47]. The average kaempferol content in juice pressed from apple of mixed varieties is 7 mg per dm^3; the figure for quercetin is 35 mg/dm^3.

The results of this research indicate that the inhibition due to kaempferol in 1 mg/100 cm^3 concentration (i.e. a concentration similar to that actually found in apple juice) amounts to about 20%, while 5 mg of quercetin per 100 cm^3 causes a roughly 18% drop of polygalacturonases activity. It is thus to be expected that the joint action of these two compounds may produce an additive or synergistic effect.

Noteworthy among the remaining polyphenols are m- and p-coumaric acids representing phenylpropenic acids. At concentrations corresponding to those actually occuring in apple juices they cause polygalacturonases activity drops of 14.0 and 18%, respectively. The other compounds, classified as phenolic acids (protocatechuic acid) and phenyl propenic acids (o-coumaric, caffeic and ferulic acids as well as chlorogenic acid which occurs in apples in the largest quantities) display minimal inhibitory capabilities in concentrations corresponding to those actually found in apple juices, and hence they do not have an adverse effect in the process of enzymatic clarification of juices.

Worth noting in the group of flavonoids is dl-catechol found in apple juice in quantities ranging from 5 to 1000 mg per dm^3, depending on the applied

technological process [2, 4]. Our studies show that already at the concentration of 5 mg/100 cm^3 this compound causes a ca. 10% inhibition, with this figure growing with the increase of concentration: 80 mg/100 cm^3 the degree of inhibition is about 55%. Given the fact that dl-catechol is particularly sesceptible to oxidation processes which increase its inhibitory effect on pectinolytic enzymes, it must be surmised that it is one of the basic inhibitors disrupting of the enzymatic processing of apple juice. The dependence of polygalacturonases inhibition by dl-catechol on this compound's concentration in the reaction mixture is illustrated in Figure.

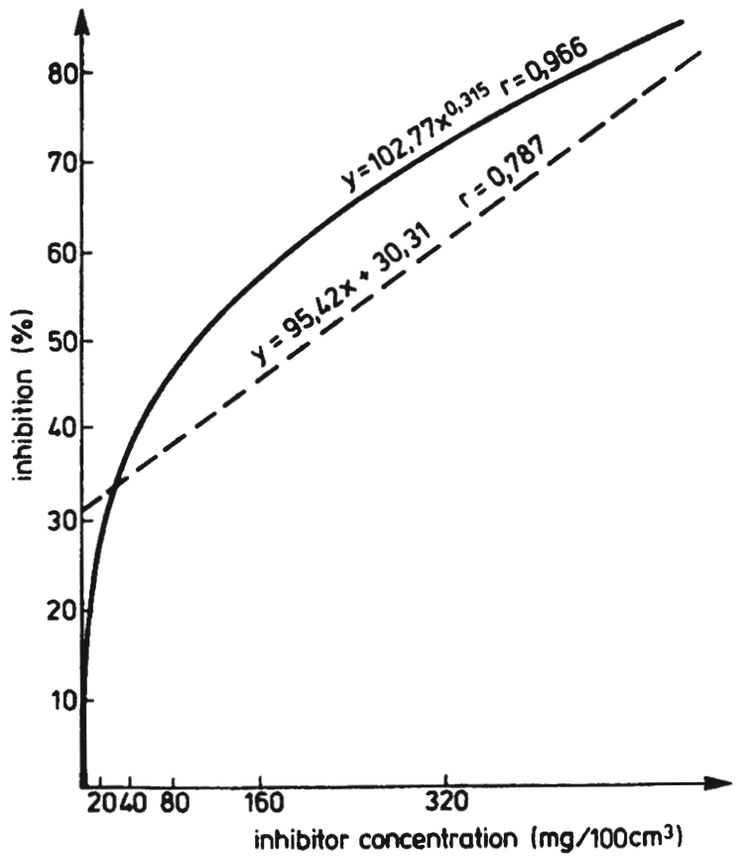

Figure. Dependence of inhibition of polygalacturonases from the Pectinase preparation on dl-catechol concentration in the medium

CONCLUSIONS

1. The susceptibility of pectinolytic enzymes to polyphenol inhibitors depend on the kind of enzyme and its purity.

2. The strongest inhibitors of pectinolytic enzymes among the 15 studied polyphenolic compounds were: dl-catechol, tannic acid, flavonols and their glicosides, kaempferol, quercetin, quercitrin and rutin and, among the phenylpropenic acids — m- and p-coumaric acids.

3. Shikimic and protocatechuic acids as well as phenylpropenic acids (ferulic, caffeic and chlorogenic) inhibit polygalacturonases to a minimal extent.

4. Ferulic acid turned out to be an effective inhibitor of pectinesterase but had no effect on the activity of polygalacturonases.

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Manuscript received: Februar, 1987
Authors address: 02-528 Warszawa, Rakowiecka 26/30

M. Mitek, B. Drzazga

WSPÓŁZALEŻNOŚĆ POMIĘDZY EFEKTEM ENZYMATYCZNEGO KLAROWANIA SOKÓW JABŁKOWYCH A ILOŚCIĄ I JAKOŚCIĄ POLIFENOLI. I. INHIBICJA ENZYMÓW PEKTYNOLITYCZNYCH PRZEZ POLIFENOLE — BADANIA NA UKŁADACH MODELOWYCH

Katedra Technologii Przemysłu Fermentacyjnego i Owocowo-Warzywnego, SGGW-AR, Warszawa

Streszczenie

Przeprowadzono badania nad wpływem 15 związków polifenolowych na aktywność poligalakturonaz oraz pektynoesterazy zawartych w wybranych preparatach pektynolitycznych: Pektopol PT, Pectinase i Panzym Super. Badania przeprowadzono na układach modelowych, stosując jako substrat jabłkowy preparat pektynowy produkcji ZPOW Jasło o stopniu estryfikacji pektyn 67,0%.

Związki polifenolowe produkcji firm zagranicznych dodawano bezpośrednio do roztworu substratu pektynowego w ilościach zbliżonych do tych, jakie występują w sokach jabłkowych (seria I badań), bądź też uprzednio przetrzymywano je z roztworem preparatu pektynolitycznego przez 30 min (seria II i III badań). Przebadano też wpływ wzrastających dawek polifenoli na aktywność PG-az, przy zachowaniu stałego stężenia substratu pektynowego (seria III badań).

Inhibicję poligalakturonaz przez polifenole oznaczano wykorzystując do pomiaru aktywności tych enzymów metodę wiskozymetryczną, a do oznaczania inhibicji pektynoesterazy — metodę miareczkowania potencjometrycznego.

Na podstawie analizy statystycznej uzyskiwanych wyników (tab. 2) spośród 15 badanych polifenoli wyłoniono 9, których działanie inhibicyjne w stosunku do poligalakturonaz okazało się

statystycznie istotne. Są to kwasy: szikimowy, o- i m-kumarowe oraz taninowy, a także kwercytryna, kwercetyna, kemferol, apigenina i rutyna. Wykazano ponadto (tab. 1), że poligalakturonazy zawarte w preparacie Pektopol PT oznaczają się większą odpornością wobec inhibitorów polifenolowych, aniżeli te same enzymy z preparatu Pectinase, a także, że w porównaniu z poligalakturonazami, pektynoesteraza okazała się mało wrażliwa na działanie polifenoli. Jedynym wyjątkiem był kwas ferulowy, co może mieć znaczenie z punktu widzenia prowadzenia procesów pektynolizy soków jabłkowych. Na podstawie wyników II serii badań /tab. 3/ wysunięto przypuszczenie, że inhibicyjny wpływ polifenoli na enzymy pektynolityczne jest efektem ich oddziaływania z jednej strony na białko enzymatyczne, a z drugiej na substrat pektynowy. Wyniki III serii badań pozwoliły określić siłę inhibicyjną polifenoli w zależności od ich stężenia, przy czym z punktu widzenia praktyki przemysłowej interesujące są te zakresy stężeń, w jakich poszczególne polifenole występują naturalnie w surowcach. W zakresie stężenia do 10 mg/100 cm^3 silnymi inhibitorami enzymów epktynolitycznych okazały się: kemferol, kwercetyna, kwercytryna, apigenina i kwasy m- i p-kumarowe, a w stężeniach powyżej 40 mg/100 cm^3 — dl-katechina. Kwasy chlorogenowy, kawowy i protokatechowy należy zaliczyć do grupy związków nie wykazujących aktywności inhibicyjnej w stosunku do enzymów pektynolitycznych.