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APPLYING OF THE BIOCHEMILUMINESCENCE METHOD IN THE DETERMINATION OF OXIDIZING ENZYMES ACTIVITY IN SOME FRUITS AND VEGETABLES

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Key words: activity of oxidizing enzymes, peroxidase, o-diphenol oxidase, ascorbate oxidase, biochemiluminescence, apples, cucumbers, potatoes.

The biochemiluminescence method was applied to determine activity of oxidizing enzymes i.e. peroxidase, o-diphenol oxidase, ascorbate oxidase in apples, cucumbers and potatoes. The results indicate the correlation between the biochemiluminescence intensity and both peroxidase activity and total activity of peroxidase and o-diphenol oxidase in apples and cucumbers.

Biochemiluminescence is a very weak visible radiation in the range of 450-600 nm which is emitted by various biological systems [19]. Homogenates of plant and animal tissues emit 10^5-10^8 quantum/g. sec [13]. Biochemiluminescence is a widespread phenomenon in living matter. It may accompany the enzymatic and non-enzymatic processes of polyphenol oxidation. The reaction mechanism is not yet exactly known. It is possible that compounds comprizing π-electrons are the source of biochemiluminescence.

In recent years luminescence has been widely introduced in the analytical procedures. The method is convenient due to its speed, simplicity and high sensitivity which cannot be achieved by any other method, for example peroxidase levels as low as 10^{-14}M have been detected [21]. Such a sensitivity can be obtained by using the method of single photon counting by scintillation counters. However, sufficient precision of measurement and a low level of errors are conditioned by strict compliance with both the reaction conditions and the method of radiation intensity determinations.

Numerous applications based on the luminescence assay have been described in literature. This method was used to determine ATP, glucose, phenolic compounds, proteins, formaldehyde and molecular oxygen [9, 18].

In agricultural diagnostics, the luminescence method was applied to investigate crop resistance to extreme weather and soil conditions [13].

Attempts have been also made to assay enzyme activities in model systems by means of luminescence. The oxidation of pyrogallol by hydrogen peroxide in the presence of horse radish peroxidase was studied. Luminescence was also applied to examine the metabolic activity of living organisms [6, 7], to estimate the quality of stored cereals [10], fodder [15] and potatoes [4]. Results of certain investigations indicate that biochemiluminescence is related only to peroxidase activity and the influence of o-diphenol oxidase is negligible and can be disregarded. However, the oxidation of ascorbic acid by assorbate oxidase is accompanied by luminescence [1].

The purpose of the present work was to investigate the possibility of applying the luminescence method to assay oxidizing enzymes in the raw and processed fruits and vegetables.

MATERIAL AND METHODS

Four varieties of apples, two varieties of cucumbers and two varieties of potatoes were used in the present work. The experimental material was chosen because of different levels of enzymes activities.

Apples of four varieties, namely Idared, Jonatan, McIntosh and Starking, were sampled monthly from the Institute of Pomology in Skierniewice after being cold stored for 2, 3, and 4 months, i.e. in November, December and January. Fruits were stored in conditions considered to be optimal [8] i.e. in an atmosphere containing 5% CO_2 and 3% O_2 at 0°C. Cucumbers var. Wilanowski cultivated in a plastic tunnel were harvested when mature for consumption on July 21 and August 2 and the outdoor grown cucumbers var. Table Green were picked up on August 18 and 26. Both varieties were obtained from the experimental fields of the Warsaw Agricultural University. Potatoes var. Baca and Fionia were harvested in October and sampled weekly for investigations for four weeks in November. All fruits and vegetables were kept in cold storage at 5-8°C.

The enzymatic activity was determined by the luminescence method. The intensity of electromagnetic radiation which arised in the oxidation of pyrogallol in the presence of plant tissue enzymes was registered. The equipment used for the luminescence measurements consisted of the high voltage power supply PZS-5, the electronic scaler PEL-+ and the scintillation unit USB-2 with a photomultiplier EMI type 9514 S. The reaction took place in a reaction cell made of inert glass Razoterm. The reaction mixture consisted of 1 cm^3 phosphate buffer 1/15 M at pH 6.98, 0.2 cm^3 1% pyrogallol and 0.2 cm^3 1% hydrogen peroxide. After having placed the cell in a reaction chamber three measurements of the intensity of the

model reaction were taken. A single measurement lasted 20 sec. Then 0.2 cm^3 of buffered enzymatic extract corresponding to 40 mg of plant tissue, was added to the reaction mixture and the luminescence intensity was registered for 200 sec. The number of pulses was read every 20 sec. The results are presented in the form of a luminescence curve showing the change of the pulse number during the reaction time and both maximal ($BChL_{max}$) and total ($BChL_{pole}$) intensity of luminescence after taking into consideration the background intensity of the system. The above method was applied to determine the radiation intensity both in the homogenates of the raw materials and in the homogenates subjected to thermal inactivation.

Activity of peroxidase and o-diphenol oxidase was also determined by the method of Willstätter and Stoll as modified by Reifer [2] and activity of ascorbate oxidase was measured by the modified iodometric method [16].

The results were statistically analysed. The correlation coefficients for the relationship between the luminescence intensity and traditionally determined activities of peroxidase, o-diphenol oxidase, total activity of these two enzymes and activity of ascorbate oxidase were calculated. In the case of a significant correlation the regresion equations were calculated by means of the method of least squares.

RESULTS AND DISCUSSION

In all the raw materials the presence of oxidizing enzymes, namely peroxidase, o-diphenol oxidase and ascorbate oxidase, was observed (Table 1).

Table 1. Activity of peroxidase (PO), o-diphenol oxidase (PFO) and ascorbate oxidase (AO)

Raw material, variety	PO mg purpurogalline/g tissue	PFO mg purpurogalline/g tissue	AO mg ascorbic acid/g tissue
Apples			
Idared	0.2-0.4*)	0.2-0.3	0.1-0.5
Jonatan	0.1-0.2	0.2-0.3	0.1-0.5
McIntosh	0.2-0.3	0.1-0.2	0.2-0.3
Starking	0.3-0.4	0.2-0.3	0.1-0.5
Cucumbers			
Wilanowski	4.5-19.0	0.2-0.3	2.8-5.6
Table Green	4.5-8.1	0.2-0.6	1.8-2.3
Potatoes			
Baca	9.6-11.4	1.6-2.1	0.3-0.9
Fionia	11.0-13.4	1.3-1.9	0.3-0.5

*) Minimal and maximal values of enzymes activity recorded in plant material in the whole period of storage

Activity of o-diphenol oxidase was the highest in potatoes, lower in cucumbers and apples. The same is true of peroxidase activity. Activity of ascorbate oxidase was the highest in cucumbers. Activity of peroxidase in all the raw materials was higher than the activity of o-diphenol oxidase.

There are many reports in literature concerning the activity of the enzymes studied. However, they usually refer to selected plant tissues and in many reports enzymatic activities have been given in differential and unconvertible units. Therefore there was no possibility to compare the results of our own experiments with the other authors' results in relation to the values of activity. Besides the application of various enzyme substrates leads to different results which has been pointed out by Chan and Yang [3], Mihályi et al. [12], Vámos-Vigyázó et al [22].

The highest luminescence intensity was found in potatoes (Table 2). Among the examined varieties of apples, Jonatan and McIntosh had a relatively low level of luminescence intensity while Idared revealed much

Table 2. The biochemiluminescence intensity of plant material

Raw material, variety	$BChL_{max}$ thousand pulses/20 sec	$BChL_{pole}$ thousand pulses/200 sec
Apples		
Idared	70-160	550-1230
Jonatan	30-60	220-510
McIntosh	20-50	160-320
Starking	40-90	310-750
Cucumbers		
Wilanowski	30-110	160-680
Table Green	50-130	330-660
Potatoes		
Baca	160-400	1150-2420
Fionia	200-450	1110-2370

higher activity. The luminescence intensity of both varieties of cucumbers remainded at an intermediate level. Typical patterns of the luminescence curves were obtained in all cases (Fig. 1 and 2). After having added the enzyme source, a sudden increase of pulses number was observed which was followed by a slow decrease due to a loss of enzyme substrate. The high luminescence intensity in the first stage of reaction was an indicator of the formation of intermediate products in the excited state [14]. The rate of ascending and descending of the curve may be changed when a different concentration and volume of reaction substrates is applied.

An attempt has been made to establish which of the oxidizing enzymes has the strongest catalyzing effect on the reaction of pyrogallol luminescence. In the varieties of apples and cucumbers used in the investigations a significant positive correlation occured between the lu-

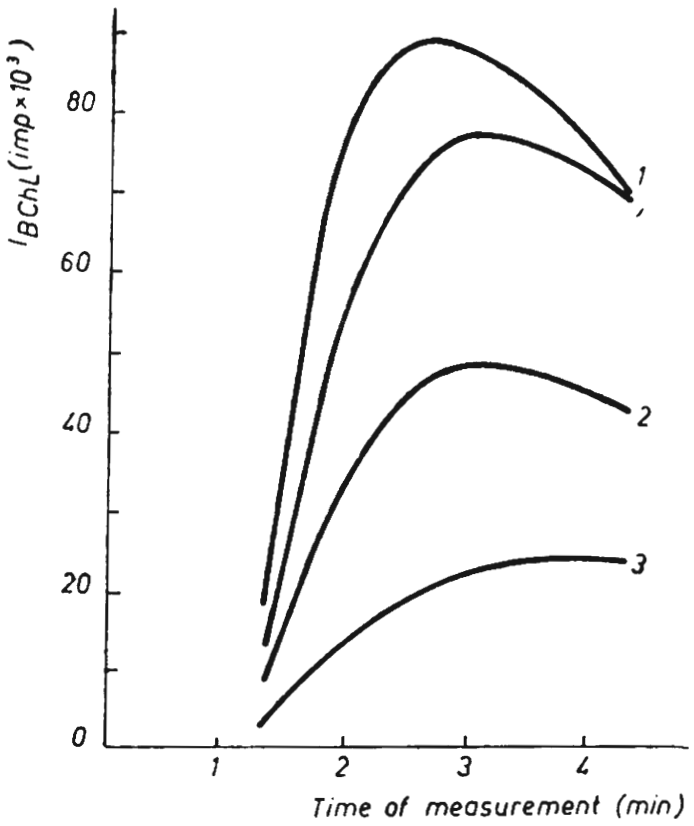

Fig. 1. Luminescence curves of four apples varieties (December); 1 — Idared, 2 — Jonatan, 3 — McIntosh, 4 — Starking

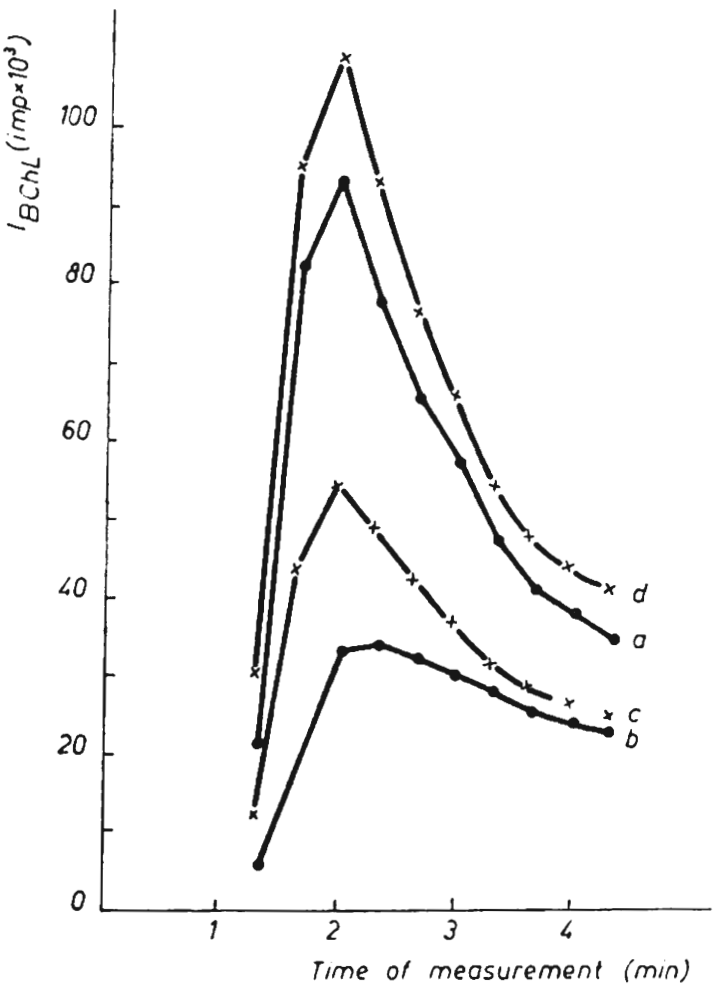

Fig. 2. Luminescence curves of cucumbers; var. Wilanowski: a — July 21, b — August 2: var. Table Green: c — August 18, d — August 26

minescence intensity and both peroxidase activity and total activity of peroxidase and o-diphenol oxidase (Table 3). Similar results were also obtained by Dziecioł and Antoszewski [5] in their work concerning the strawberry receptacle. Previous model studies also confirm this statement. However, it cannot be referred to all plant materials. A negative correlation between the luminescence intensity and peroxidase activity was obtained in potatoes, a fact which is difficult to explain. Besides peroxidase, there are probably others factors in the tissue of plants, which may determine pyrogallol luminescence, for example lipids present in mitochondria and lysosomes. Oxidation of lipids by molecular oxygen after a previous destruction of the combination of lipid and protein, leads to a chain reaction. The photon emission during de-excitation is a result of this reaction [17]. Luminescence may also accompany oxidation processes (both enzymatic and non-enzymatic) of phenolic compounds which can be released from their glycosides as a result of structure destruction or disturbance of tissue metabolism [20].

Table 3. Correlation coefficients for relationship between the maximal luminescence intensity ($BChL_{max}$) and oxidizing enzymes activity determined by chemica methods

Raw material, variety	PO+PFO	PO	PFO	AO
Apples	0.60**)	0.52*)	0.51	
Cucumbers	0.94*)	0.94*)		0.93*)
Table Green	0.97**)	0.98**)		
Potatoes				
Baca	−0.99**)	−0.96**)		
Fionia				0.99**)

*) Means significant correlation with P = 90%
**) Means significant correlation with P = 95%

In apples a significant correlation was also observed between the luminescence intensity and activity of o-diphenol oxidase. The positive correlation between the luminescence intensity and ascorbate oxidase activity which was obtained in cucumbers of Wilanowski variety and in potatoes of Fionia variety confirms the model studies carried out by Bogdański and Grabiec [1].

In no case a significant multiple correlation between the luminescence intensity and activity of all examined enzymes was obtained.

This means that there is no simulaneous correlation between biochemiluminescence intensity and the activity of peroxidase o-diphenol oxidase and ascorbate oxidase.

The thermal inactivation of enzymes proceeded at different speeds in the tissues examined. Even in the same variety of plant at a higher

initial activity a slower proportional decrease of the luminescence intensity was observed, a fact already pointed out in previous studies [11]. Destruction of 99% of enzymatic activity in apples required heating for 5, 10 or 30 minutes at 80°C, depending on the variety. In cucumbers, 99% of enzymatic activity was inactivated by heating for a time shorter than 5 secs at 80°C, 5 minutes at 75°C, 10 minutes at 70°C or 30 minutes at 65°C. In potatoes the time necessary to inactivate 99% of enzymatic acivity at 80°C was shorter than 5 secs, 5 to 30 seconds (depending on the variety) at 75°C more than 2 minutes at 70°C.

Short heating at high temperature is favourable for a reactivation of the enzyme. This phenomenon was observed in boiled enzymatic extracts whose luminescence intensity was higher than the intensity of extracts subjected to long heating at lower temperature. The phenomenon of residual peroxidase activity in raw materials subjected to long-lasting heat treatment has not yet been explained though it has been known for quite a long time. According to Winter [23] it is caused by a combination of the prosthetic group of degradating enzyme (protohemin) and denaturated enzymatic protein. This combination effectively protects inactivated peroxidase agains further degradation.

In all inactivated materials, qualitative tests with benzidine were carried out to determine peroxidase presence. The results of these experiments were compared with the total luminescence intensity. In all cases the qualitative tests gave a negative result when the total luminescence intensity was lower than 10 000 pulses. This number of pulses was always below 2% of initial activity. Positive results indicating the presence of peroxidase were obtained when the luminescence intensity was higher than 14 000-15 000 pulses in 200 secs. Therefore the measurements of luminescence intensity may serve as a reliable indicator of pasteurization or another type of heat treatment when the system of oxidizing enzymes should be destroyed.

CONCLUSIONS

1. A significant positive correlation was found between enzymatic activity determined by the luminescence method and both peroxidase activity and total activity of peroxidase and o-diphenol oxidase.

2. The luminescence method of determination of oxidizing enzymes may be applied to observe thermal enzymes inactivation in various fruits and vegetables.

3. No significant multiple correlation occured between the luminescence intensity and activity of all examined oxidizing enzymes.

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WYKORZYSTANIE BIOCHEMILUMINESCENCJI DO OKREŚLANIA AKTYWNOŚCI NIEKTÓRYCH ENZYMÓW OKSYDOREDUKCYJNYCH W WYBRANYCH OWOCACH I WARZYWACH

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Streszczenie

Dokonano enzymatycznej analizy jabłek odmian Idared, Jonatan, McIntosh i Starking, ogórków odmian Wilanowski i Table Green oraz ziemniaków odmian Baca i Fionia. Jabłka badano po dwóch, trzech i czterech miesiącach przechowywania w kontrolowanych warunkach (temperatura 0°C, 5% CO_2, 3% O_2). Każdą z odmian ogórków

zbierano dwukrotnie w stadium dojrzałości konsumpcyjnej. Badanie ziemniaków prowadzono podczas przechowywania przez miesiąc w odstępach tygodniowych.

Do badania aktywności enzymatycznej zastosowano metodę biochemiluminescencyjną. Rejestrowano natężenie promieniowania elektromagnetycznego powstającego w wyniku reakcji utleniania pirogalolu przez enzymy obecne w badanych tkankach roślinnych. Ponadto oznaczano metodami konwencjonalnymi aktywność peroksydazy, oksydazy o-dwufeolowej i oksydazy askorbinianowej.

Najwyższą aktywność oksydazy o-dwufenolowej oraz peroksydazy stwierdzano w ziemniakach, a oksydazy askorbinianowej w ogórkach. Również ziemniaki wykazywały najwyższą spośród badanych surowców impulsogenność.

Przeprowadzone obliczenia statystyczne wskazują, że w badanych odmianach jabłek i ogórków istnieje istotna dodatnia korelacja między natężeniem biochemiluminescencji a łączną aktywnością peroksydazy i oksydazy o-dwufenolowej oraz między natężeniem biochemiluminescencji a aktywnością peroksydazy. Istotną zależność natężenia biochemiluminescencji od aktywności oksydazy o-dwufenolowej uzyskano w jabłkach, a w ogórkach i ziemniakach dodatnio skorelowane były natężenie biochemiluminescencji i aktywność oksydazy askorbinianowej.