Beiträge zur Tabakforschung International/*Contributions to Tobacco Research* Volume 24 · No. 1 · April 2010

DOI: 10.2478/cttr-2013-0877

Biochemical Studies on Curing and Fermentation Processing Periods of Different Tobacco Plant (*Nicotiana tabacum* L.) Cultivars*

by

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SUMMARY

We studied the changes in the activity of the enzymes polyphenol oxidase (PPO) and peroxidase (POX), the concentration of total soluble phenolic compounds and soluble protein content in different tobacco cultivars (Virginia and Burley) during curing. The curing procedure was a special combination of open-air-curing and flue-curing methods and a long fermentation period. In the curing period the changes in data suggested a combination of the biochemical processes and the direct effect of oxygen. A slight increase then a decrease in the concentrations of both total soluble phenolic compounds and the soluble protein content were detected. In this period we found no correlation between the concentration of total soluble phenolic compounds, the decreasing PPO and the increasing POX activity. In the fermentation period a deactivation of the enzymes (PPO and POX) and a decrease in the concentration of both total soluble phenolic compounds and soluble protein content were found, because the fermentation is not a biochemically-regulated process. Moreover, these results suggest that the end of curing period is the most favourable time for protein isolation from different tobacco cultivars. [Beitr. Tabakforsch. Int. 24 (2010) 24–28]

ZUSAMMENFASSUNG

Wir haben die Aktivitätsveränderungen der beiden Enzyme Polyphenoloxidase (PPO) und Peroxidase (POX), sowie die Konzentration der gesamten löslichen phenolischen Verbindungen und löslichen Proteine in verschiedenen Tabaksorten (Virginia und Burley) während des Curings untersucht. Das Curing war eine spezielle Kombination von Open-Air-Curing, Flue-Curing Methoden und einer langen Fermentationsperiode. Die Veränderungen der Aktivitäten während der Curing-Periode weisen auf eine Kombination von biochemischen Prozessen und den direkten Einfluss von Sauerstoff hin. Es konnte ein leichter Konzentrationsanstieg, gefolgt von einer Abnahme der löslichen phenolischen Verbindungen und der löslichen Proteine detektiert werden. Während dieses Zeitraumes konnte kein Zusammenhang zwischen der Konzentration der löslichen phenolischen Verbindungen, dem Abfall der PPO-Aktivität und dem Anstieg der POX-Aktivität gefunden werden. Während der Fermentationsperiode konnte eine Deaktivierung der beiden Enzyme PPO und POX sowie eine Konzentrationsabnahme der löslichen phenolischen Verbindungen und der löslichen Proteine gefunden werden, da die Fermentation kein biochemisch regulierter Prozess ist. Darüber hinaus geben die Daten den Hinweis, dass der geeignete Zeitpunkt für eine Proteinisolierung bei den verschiedenen Tabakkulturen das Ende der Curing-Periode ist. [Beitr. Tabakforsch. Int. 24 (2010) 24–28]

RESUME

Nous avons étudié les changements dans l'activité des enzymes polyphénol oxydase (PPO) et péroxydase (POX), la concentration en composés phénoliques solubles totaux et la teneur en protéines solubles de différentes variétés de tabac (Virginie et Burley) au cours du séchage qui a consisté en une combinaison particulière de méthodes de séchage en plein air et de séchage à l'air chaud et d'une longue période de fermentation. Lors du séchage, les changements dans les données suggèrent une combinaison de processus biochimiques et un effet direct de l'oxygène. Une légère augmentation suivie d'une diminution des concentrations des deux composés phénoliques soluble totaux et de la teneur en protéines solubles a été détectée.

*Received: 16th February 2009 – accepted: 23rd April 2009

Au cours de cette période, nous n'avons trouvé aucune corrélation entre la concentration de composés phénoliques solubles totaux et la baisse des PPO ainsi que l'augmentation de l'activité des POX. Pendant la fermentation, nous avons constaté une désactivation des enzymes (PPO et POX) et une baisse des concentrations des deux composés phénoliques solubles totaux et de la teneur en protéine soluble car la fermentation n'est pas un processus biochimiquement régulé. Toutefois, ces résultats suggèrent que la fin de la période de séchage est la plus favorable à l'isolation de protéines des différentes variétés de tabac. [Beitr. Tabakforsch. Int. 24 (2010) 24–28]

INTRODUCTION

Tobacco plant (*Nicotiana tabacum* L.) is used not only in the tobacco industry but also for other purposes. Therefore, the biochemical study of different important tobacco components could be useful. Polyphenols and pigments are components influencing tobacco quality and usability. It was presumed that the study of the enzyme activity taking part in the metabolism of polyphenols and pigments (e.g. polyphenol oxidase) could be informative about the quality of tobacco plant and products. The concentration of phenol derivatives in tobacco plant is determined by the balance of their synthesis and degradation (1).

Polyphenol oxidases (PPO, EC 1.14.18.1) are copper enzymes using molecular oxygen in the catalysis of the oxidative degradation of dihydroxybenzol (pyrocatechol and hydroquinon) derivatives (synthesized by the oxidation of phenol) to quinones, that can polymerize spontaneously to different pigments (e.g. melanines). These processes can be useful in tobacco (by enrichment in colour and quality agents). On the other hand it can cause a deterioration in nutrient quality of fruits and vegetables (by enzymatic browning) (2). In the case of various stress conditions in the accumulation and oxidation of different polyphenol derivatives undesirable toxic active oxygen species could form therefore the activity of special enzymes, above all peroxidases (POX, EC 1.11.1.7) for the degradation of these dangerous species was also increased (3).

Tobacco leaves as well as other higher plant leaves contain soluble and insoluble proteins which are usually equal in quantity. It was found that the water soluble protein fraction of tobacco leaves is well balanced containing high levels of essential amino acids, therefore it is considered a protein source in nutrition (4). After isolation of soluble proteins the residual leaf material can be used in the tobacco industry (5).

We found only a few publications about biochemical studies on growing tobacco plants and on tobacco leaves during and after curing processes. The possibility of the synthesis of red pigments in flue-cured tobacco by biochemical and chemical methods was studied by means of PPO preparations isolated from green tobacco leaves (6). The changes of phenol derivatives in tobacco leaves during flue-curing process were studied by Chinese researchers who found a rapid enhance in the first 24 h of curing then a decrease until 72 h. At the end of curing an increase again was detected. The changes in PPO and POX activity were the opposite (7). Earlier we studied the changes in the activity of enzymes PPO and POX, the concentration of total soluble phenolic compounds and soluble protein content of different tobacco cultivars (Virginia and Burley) measured in tobacco plants during cultivation. We found some correlation between the concentration of total soluble phenolic compounds and PPO activity data (8).

Now we studied the changes in the activity of enzymes PPO and POX, the concentration of total soluble phenolic compounds and soluble protein content of different tobacco cultivars during a curing process, which was a special combination of open-air-curing and flue-curing methods followed by a long fermentation period.

MATERIALS AND METHODS

Materials

The green upper and bottom leaves of tobacco cultivars (*Nicotiana tabacum* L.) Virginia and Burley were given by V-Tabak Hungarian Tobacco Manufacturing Company, Szolnok, Hungary in the autumn of 2005 (30th September) and the curing was started on the same day. For biochemical studies the tobacco leaves were homogenized and the extracts (0.10 g/mL in water after centrifugation for 10 min at 4 °C at 10,000 rpm) were made. Chemicals were purchased from SIGMA-ALDRICH Co. and REANAL Finechemical Co. (Budapest, Hungary).

Methods

The first step of tobacco leaf processing is the curing. There are two different types of drying of tobacco leaves as far as the applied curing technologies are concerned: open-air-curing and flue-curing. Open-air-curing is also referred to as „natural" curing because environmental conditions during curing are largely determined by the prevailing weather. On the premises of flue-curing the circumstances are primarily controlled by the air temperature and the amount of dry air that passes through the mass of tobacco (1). Our model system was a special combination of open-air-curing and flue-curing methods: tobacco leaves (upper and bottom leaves separately) were dried in a special store-room for four weeks under controlled circumstances (20–25 °C and 85–90% relative humidity) without passing air through the leaves.

The second stage of tobacco leaf processing was the fermentation. In our eighteen-week long model system bales containing a mixture of upper and bottom tobacco leaves in agglomerated form were stored under controlled circumstances (20–25 °C and 85–90% relative humidity). In our laboratory fermentation model the tobacco leaves were agglomerated by hand-press, therefore the presence of air in bales was considerable. The samples were taken from the inside part of the bales and not only the activity of enzymes PPO and POX, the concentration of total soluble phenolic compounds and soluble protein content, but also the pH values of the samples were measured.

Samples were taken every week during the curing and fermentation periods. Originally for the samples complex extraction methods were generally used by Tris-acetate buffer and surfactants followed by a partial purification

before the analysis of the samples (9). But earlier we found that because of the presence of the hydrophobic components in food industrial raw materials of plant origin a concentrated aqueous solution (0.50 g/mL in water) could be used for the measurement of the activity of enzymes with acidic and neutral pH optima and the total phenol content of the samples without previous purification (10). Because of the relatively low water content of tobacco plant (lower than in fruits) aqueous solutions were only 0.10 g/mL in water. Data measured of concentrated aqueous extractions were compared to data of other extractive agents described in the literature and no significant differences were found.

POX activity of the extracts was determined using *o*-dianisidine as hydrogen donor in sodium acetate (pH 5.1) (11). One unit of POX activity was defined as the amount of enzyme that caused decomposition of 1 µmol/min hydrogen peroxide in the reaction mixture (1.0 ml).

PPO activity was detected in sodium acetate (instead of sodium phosphate) buffer (pH 6.5) using pyrocatechol substrate (12). One unit of PPO activity was defined as the amount of enzyme that caused 0.01 unit/min of change in absorbance of the reaction mixture (1.0 ml) at 420 nm.

Total phenol content was measured by the colorimetric method with Folin & Ciocalteu's phenol reagent (13) and the results were expressed in Gallic Acid Equivalent (GAE) value (mmol/g dry weight of tobacco leaves).

Protein concentration was measured by LAYNE method (14). Data of samples were calculated on the dry weight of leaves. Water content of samples was determined by Sartorius M 50 Aquatest instrument. The results are given as the average values of four replications with a standard deviation ±5%.

RESULTS AND DISCUSSION

The results of our combined curing model system

In the concentration of total soluble phenolic compounds expressed in GAE value of tobacco leaves in curing we found a slight increase from 30th September to 7th October both in upper and bottom leaves followed by a decrease until 21st October (Figure 1). The increase was attributed to the hydrolysis liberating of soluble phenolic compounds from their fixed insoluble forms. The decrease of total soluble phenolic compound content was attributed to their oxidative degradation to synthesize of colouring agents.

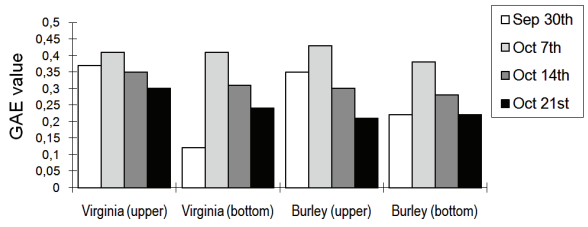


Figure 1. Changes in the concentration of total soluble phenolic compounds. Expressed in GAE value (mmol gallic acid/g dry tobacco) in upper and bottom leaves of Virginia and Burley tobacco cultivars.

In PPO activity of tobacco leaves in curing at the start of our studies (30th September) we found a high activity in upper leaves (the younger) and low activity in bottom leaves (the older). In upper leaves we measured higher activity for Burley than for Virginia. Then a radical decrease in both upper and bottom leaves was detected (Figure 2). We found no correlation between the concentration of total soluble phenolic compounds and the PPO activity data. This suggests a direct oxidation of phenolic compounds instead of enzymatic oxidation catalysed by PPO in curing.

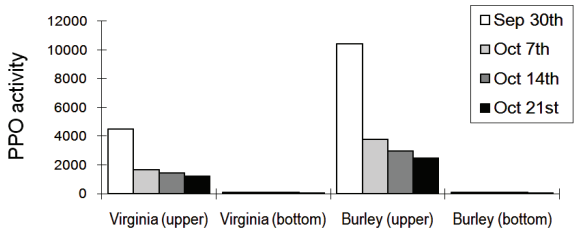


Figure 2. Changes in PPO activity (U/g dry tobacco) in upper and bottom leaves of Virginia and Burley tobacco cultivars

The continuous increase in POX activity of tobacco leaves in curing could be characteristic for a stress situation (Figure 3). Similar deviation was found earlier in change of POX and PPO activity of tobacco leaves during cultivation (8). We measured higher activity for Virginia than for Burley.

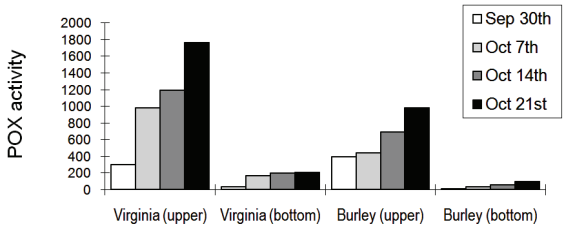


Figure 3. Changes in POX activity (µmol/min * g dry tobacco) in upper and bottom leaves of Virginia and Burley tobacco cultivars

In the tobacco industry protein concentration is calculated generally in an indirect way on the basis of total nitrogen content or by amino acid analysis. Apart from protein nitrogen the total nitrogen content contains also alkaloid and nitrate nitrogen content. Now the soluble protein concentration of tobacco samples was determined directly by the LAYNE method using biuret for calibration (14).

In the concentration of soluble protein content of tobacco leaves in curing we found a continuous enhance in upper leaves and we detected slightly higher values for Virginia than for Burley. In bottom leaves we found a slight enhance from 30th September to 7th October for Virginia and from 30th September to 14th October for Burley followed by a decrease until 21st October (Figure 4). The increase was attributed to the partial hydrolysis of insoluble protein fraction and the decrease in bottom leaves to the further hydrolysis of soluble proteins to amino acids.

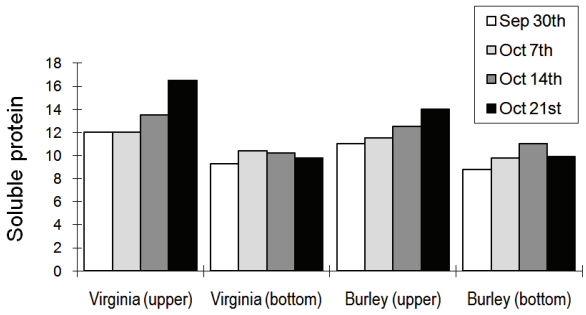


Figure 4. Changes in soluble protein content (mg protein/g dry tobacco) in upper and bottom leaves of Virginia and Burley tobacco cultivars system

The results of our twenty-week long fermentation model system

We found a slight decrease (from 5.5 to 5.1) in pH values of Virginia samples and a slight enhance (from 6.1 to 6.5) in Burley samples (Figure 5).

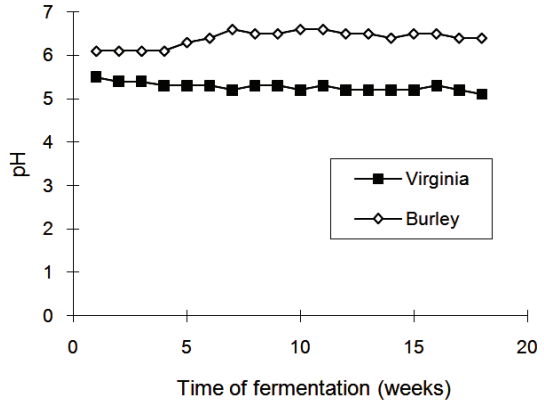


Figure 5. Changes in pH values in the bales of Virginia and Burley tobacco leaves

In the concentration of total soluble phenolic compounds expressed in GAE value of tobacco leaves we found a continuous decrease in our fermentation model (Figure 6). Higher values were detected for Virginia than for Burley.

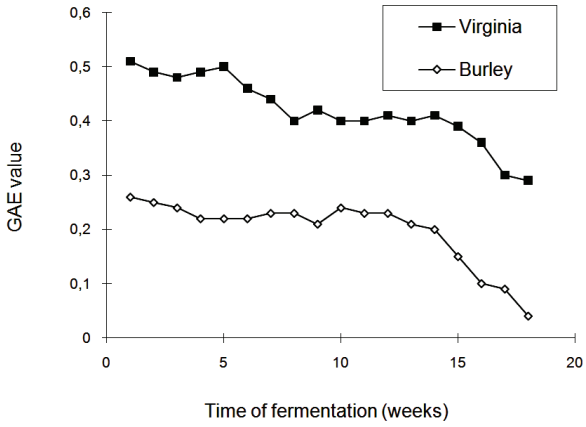


Figure 6. Changes in the concentration of total soluble phenolic compounds. Expressed in GAE value (mmol gallic acid/g dry tobacco) in the bales of Virginia and Burley tobacco leaves

We suppose that during fermentation the presence of air was enough for the oxidative degradation phenolic compounds.

We found a further radical decrease in PPO activity of tobacco leaves in our fermentation model (Figure 7). This fact suggests a direct oxidation of phenolic compounds instead of enzymatic oxidation catalysed by PPO not only in curing but also in fermentation models. We found a rapid decrease in POX activity of tobacco leaves during fermentation. The POX activity values diminished to zero practically in three weeks. The deactivation of the enzymes (PPO and POX) taking part in the elimination of not only oxygen molecules but the dangerous oxygen species (e.g. hydrogen peroxide) forming in stress situations could be characteristic for the collapse of the regulation system in tobacco leaves during fermentation.

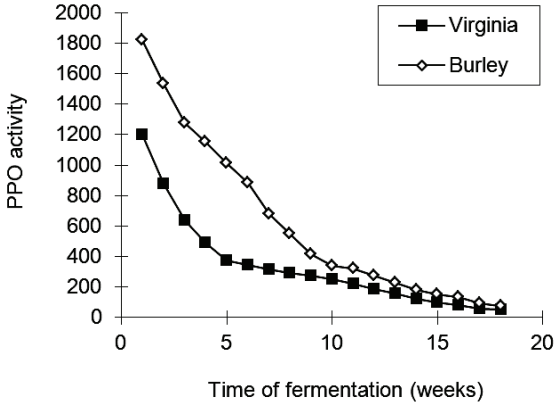


Figure 7. Changes in PPO activity (U/g dry tobacco) in the bales of Virginia and Burley tobacco leaves

Because of the further hydrolysis of soluble proteins to amino acids we found a continuous decrease in the concentration of soluble protein content of tobacco leaves in our fermentation model (Figure 8). The difference in the rate of the hydrolysis in different tobacco cultivars could be attributed to the differences in their pH values.

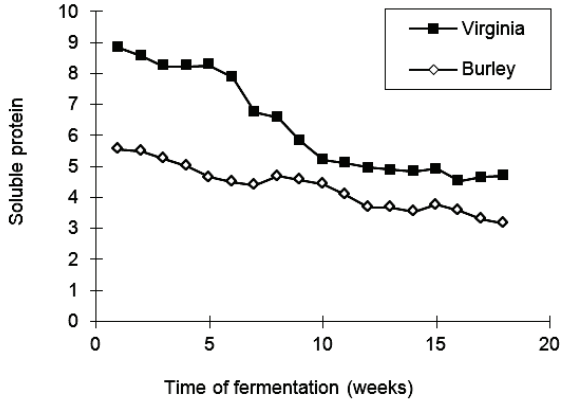


Figure 8. Changes in soluble protein content (mg protein/g dry tobacco) in bales of Virginia and Burley tobacco leaves

CONCLUSIONS

We studied the changes in the activity of the enzymes PPO and POX, the concentration of total soluble phenolic compounds and soluble protein content in different tobacco cultivars (Virginia and Burley) during curing. The curing procedure was a special combination of open-air-curing and flue-curing methods and a long fermentation period.

In the curing period the changes in data suggested a combination of the biochemical processes and the direct effect of oxygen. In the concentration of total soluble phenolic compounds the slight increase was attributed to the enzymatic hydrolysis liberating soluble phenol derivatives from their fixed insoluble forms. The following decrease was attributed to their oxidative degradation forming coloured compounds. In this period we found no correlation between the concentration of total soluble phenolic compounds and the decreasing PPO activity data. This fact suggests a direct oxidation of phenolic compounds instead of enzymatic oxidation catalysed by PPO in curing. The continuous increase of POX activity in tobacco leaves could be characteristic for the intensive presence of oxygen, because this high concentration can promote the formation of dangerous oxygen species (e.g. hydrogen peroxide).

The slight increase then a decrease in the soluble protein concentrations in the curing period could be attributed to the partial hydrolysis of insoluble protein fraction followed by the further hydrolysis of soluble proteins to amino acids. We found higher soluble protein concentrations in Virginia than in Burley. The difference in the rate of the hydrolysis in different tobacco cultivars could be attributed to the differences in their pH values.

In fermentation period the deactivation of the enzymes (PPO and POX) taking part in the elimination of oxygen and the dangerous oxygen species (e.g. hydrogen peroxide) forming could be characteristic for the collapse of the regulation system in tobacco leaves. The decrease in the concentration of both total soluble phenolic compounds and soluble protein content could be attributed to their advanced oxidative degradation during fermentation. These results support that the fermentation is not a biochemically-regulated process. We found that the end of curing period is the most favourable time for protein isolation from different tobacco cultivars.

ACKNOWLEDGEMENTS

We would like to thank the Hungarian Scientific Research Fund (OTKA K63162) for supporting this research and Z. Tar for technical assistance.

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