BIOCHEMICAL AND MICROBIOLOGICAL CHARACTERIZATION OF THE QUALITY AND USABILITY OF ROW MATERIALS OF TOBACCO ORIGIN

Theses of the doctoral dissertation of

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1. PRELIMINARIES AND OBJECTIVES OF THE RESEARCH

1.1 Preliminaries of the research

Tobacco plant (*Nicotiana tabacum* L.) is one of the ancient consumer goods. It was named by Linné and its cultivation and preparation have been a state monopoly since 1867 in Hungary. Tobacco is popular because of its alkaloids, among them nicotine that can produce both calmative and exciting effects. In the last decades the technology of tobacco processing has markedly changed. The period of the tobacco fermentation was shortened because of new technologies, and their environmental conditions became better controlled and regulated than they were earlier. The appearance of new varieties of tobacco cultivars and the changes mentioned above in the tobacco industry required the study of the properties of the quality of these varieties, and their changes during different steps of tobacco leaf processing before the industrial process. This study requires not only industrial but biochemical and microbiological approaches, as well.

These examinations can be useful not only in the tobacco industry but also in other uses of tobacco plants. Nicotine in isolated form is a component of sprays in plant protection and this compound is an important agent in the study of different nerve-cell receptors. During smoking active carcinogenic agents can be produced in tobacco products, therefore smoking is considered a dangerous health risk agent. Because of these facts the importance of tobacco industry could be decreased, therefore other uses of tobacco plants could come to the fore, for example the use of their protein content.

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, Nowadays high protein content, especially soluble protein fraction (F1 and F2 proteins) of the tobacco plant is considered a protein source in nutrition as a supplement. F1 protein is an enzyme, called RUBISCO (ribulose 1,5-bisphosphate carboxylase-oxygenase) and F2 protein is a mixture of soluble proteins of both cytoplasmic and chloroplast origin. After isolation of soluble proteins the residual leaf material could be used in the tobacco industry.

In the dissertation different samples from different tobacco plants were characterized by biochemical and microbiological methods. The department (Department of Grain and Industrial Plant Technology, Faculty of Food Sciences, Corvinus University of Budapest) has a long tradition in the study of different tobacco leaves and also has a close connection with the representatives of the Hungarian tobacco industry.

My supervisor (Professor Judit Kosáry DSc) has being studied the possibility of the characterization of different raw materials of plant origin for more than ten years at the Department of Applied Chemistry, Faculty of Food Sciences, Corvinus University of Budapest. It was found that there are protective enzymes (e.g. peroxidase, polyphenol oxidase and lipoxygenase) against abiotic and biotic stress types during the vegetation period and then storage, and the activity of these enzymes can influence the quality of raw materials of plant origin. Professor Kosáry has published a series of articles (ten) in the periodical Olaj, Szappan, Kozmetika with title *Changes in the composition of lipoxygenase isoenzymes in plants* and there are at least ten publications in other periodicals both in Hungarian and English. She had the financial support of the Hungarian Scientific Research Fund (OTKA K63162) for this topic (2006-2009).

1.2 Objectives of the research

This dissertation covers the studies of both Virginia (three of varieties) and Burley (five of varieties) tobacco cultivars (*Nicotiana tabacum* L.) presented by V-Tabak Hungarian Tobacco Manufacturing Company, Szolnok, Hungary and they were grown by me in Budapest (Hungary). These tobacco leaves were examined by biochemical and microbiological methods during their cultivation, curing and fermentation period. Tobacco leaves grown and cured by V-Tabak Hungarian Tobacco Manufacturing Company, Szolnok were studied during an industrial fermentation period, as well. All varieties of tobacco leaves mentioned above were examined by microbiological methods during their fermentation period. On the basis of the earlier results in the Department of Applied Chemistry (and in other Departments in part) the specific activity of the PhD candidate Ildikó Szedljak can be divided into seven main objectives:

- The biochemical studies of eight varieties of Virginia and Burley cultivars mentioned above were planned during own cultivation. Biochemical examinations of the activity of so-called stress enzymes (peroxidase, polyphenol oxidase and lipoxygenase), the level of the water soluble polyphenol content (characteristic for the quality of tobacco) and the level of the water soluble protein content (characteristic for the possible use of tobacco for food industry) could hardly be found in the literature.
- The biochemical examination of tobacco leaves in different positions was planned.
- I planned to study the possibility of a convergence between the lipoxygenase isoenzyme compositions of different varieties of tobacco.
- Development of a new curing system that was a special combination of open-air-curing and flue-curing methods was planned – especially for Virginia varieties of high sugar content.

- During the biochemical examinations I planned to find the best parameters of cultivation and curing to reach optimal water soluble protein content.
- Comparative biochemical and microbiological studies of the effect of open-air-curing and own combined-curing methods on the fermentation period of tobacco leaves were planned.
- Comparative biochemical and microbiological studies were planned during the fermentation period between own and industrial cultivations of tobacco plants.

2. MATERIALS AND METHODS

The experimental part of the research was performed in the laboratory of the Department of Applied Chemistry (head of department Dr. Péter Fodor). Some examinations were done at the Department of Grain and Industrial Plant Technology (acting head of department Dr. László Somogyi), at the Department of Microbiology and Biotechnology (head of department Dr. Anna Maráz) and in the laboratory of V-Tabak Hungarian Tobacco Manufacturing Company, Szolnok.
For biochemical studies three individual extracts were made in the case of all samples and three different determinations were made from the same extract with a standard deviation ±5%. To estimate the effect of the position of leaves and the types of tobacco plants on the values of the biochemical parameters two-way analysis of variance (ANOVA) without repetition was used.

2.1 Study of own cultivation of tobacco plant

Tobacco leaves were examined by biochemical and microbiological methods during their cultivation, curing and fermentation period.

Cultivation period

Both Virginia (three of varieties) and Burley (five of varieties) tobacco cultivars (*Nicotiana tabacum* L.) presented by V-Tabak Hungarian Tobacco Manufacturing Company, Szolnok, Hungary were used. The variety-names are artificial names given by the business-management of the Company in these double blind tests. These varieties are cultivated widely in Hungary. Tobacco seedlings were grown in a seedbed, and then they were transplanted into individual plastic plant bags containing a special soil mixture, and they were growing in open-air conditions. The period from the transplantation to the first sampling was called ‘precultivation period’ (69 days). Later on the dates of sampling were: 1., 9., 13., 21., 27., 36., 44. and 50. day after the precultivation period. For biochemical studies the tobacco leaves of different positions were homogenized and extracts were made.

Curing period

The new curing system that was a special combination of open-air-curing and flue-curing methods was planned – especially for Virginia varieties of high sugar content. Tobacco leaves were cured in a special store-room for four weeks in a controlled way (20-25 ^{o}C and 85-90% relative humidity) without passing air through the leaves. The dates of sampling were: 7., 14., 21. and 28. day of curing. Later on natural curing was also used.

Fermentation period

In the eighteen-week long model system bales containing a mixture of middle tobacco leaves in agglomerated form were stored under controlled circumstances (20-25 ^{o}C and 85-90% relative humidity). In this 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 in every week for biochemical studies; and at 1., 70. and 126. day for microbiological studies.

Biochemical studies

Biochemical examinations of the activity of so-called stress enzymes (peroxidase, polyphenol oxidase and lipoxygenase), the level of the water soluble polyphenol content (characteristic for the quality of tobacco) and the level of the water soluble protein content (characteristic for the possible use of tobacco for food industry) were carried out by methods described in the literature.

2.2 Study of the fermentation of the industrial cultivation of tobacco plant

After the industrial cultivation of tobacco plants the middle leaves were hung to dry in an air-curing barn (natural curing) for Burley, but for Virginia a flue-curing method was used. The samples were examined both by biochemical methods and instrumental analysis (especially for sugar content).

2.3 Microbiological studies

Microbiological studies were carried out for the fermentation period of both own cultivated and industrial cultivated tobacco leaves. These examinations were measured at the Department of Microbiology and Biotechnology for the micro organisms: mesophile aerobic microbes (among them sporeforming bacteria, and fungi – moulds and yeasts); and mesophile anaerobic microbes. In the case of moulds a determination of species was also done.

3. RESULTS AND DISCUSSION

3.1 Study of own cultivation of tobacco plant

Only a few publications about the biochemical studies on growing tobacco plants and on tobacco leaves during and after curing processes could be found in the literature. The biochemical parameters (the activity of enzymes polyphenol oxidase, lipoxygenase and peroxidase, the water soluble polyphenol content and water soluble protein content) of different tobacco cultivars were measured during cultivation and curing. Comparative biochemical and microbiological studies were made during fermentation.

3.1.1 Biochemical studies on tobacco leaves of own cultivation during growing

Two varieties of both Virginia (V-TMC1, V-TMC2, V-TMC3) and Burley (B-TMC1, B-TMC2, B-TMC3) tobacco cultivars (*Nicotiana tabacum L.*) (V-Tabak Hungarian Tobacco Manufacturing Company, Szolnok, Hungary) were used. These variety-names are artificial names given by the business-management of the Company.

Peroxidase

The tendency in changes of peroxidase activity of tobacco leaves was deviated from the results of the water soluble polyphenol and polyphenol oxidase activity and could be in connection with the age of the plant. The maximum values were found earlier in bottom leaves than in upper leaves both in Virginia and Burley varieties. The maximum data of upper leaves were higher in Virginia than in Burley varieties and almost the same in bottom leaves. It is supposed that Virginia varieties are more sensitive for the change of temperature and rainfall than Burley varieties.

Polyphenol oxidase

Polyphenol oxidases use molecular oxygen in the catalysis of the oxidative degradation of diphenols (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). At the start of the studies high polyphenol oxidase activities for both Virginia and Burley varieties (higher in Virginia than in Burley) in upper leaves and slightly less activities in middle and bottom leaves were found. Then a significant decrease in tobacco leaves of different positions was detected. There was some correlation between the concentration of water soluble polyphenol content and polyphenol oxidase activity data. In the increasing and maximum period of the water soluble polyphenol content the

polyphenol oxidase activity was high. But later because of decrease of substrate phenol content the activity of polyphenol oxidase also decreased.

Lipoxygenase

One of my objectives was to study the possibility of a convergence between the lipoxygenase isoenzyme compositions of different varieties of tobacco. The isoenzyme composition lipoxygenases can be deduced from pH dependence of the lipoxygenase activity. Contrary to other plants (e.g. apple, onion, etc.) no differences were found in lipoxygenase isoenzyme compositions of Virginia and Burley varieties. It is presumed that tobacco lipoxygenase cannot be used for this kind of distinction.

Water soluble polyphenol content

The concentration of water soluble polyphenol content is expressed in GAE value (mmol gallic acid dry tobacco g^{-1}) of tobacco leaves we found an enhance then a decrease until a quasi-constant level. This was attributed to the balance of the synthesis of phenols and then *via* their degradation the synthesis of colouring agents. We found higher values for both Virginia and Burley varieties in upper leaves (the younger) than middle and bottom leaves (the older). The higher maximum of water soluble polyphenol content in Burley varieties than in Virginia varieties suggests an intensive synthesis of colouring agents in Burley.

Water soluble protein content

In the increasing and maximum period of water soluble protein content of tobacco leaves of various Virginia and Burley varieties the dispersion of data measured was slightly higher and maximum period was slightly later for upper and middle leaves and longer for bottom leaves both in Virginia and Burley varieties than in the case of other parameters. The maximum data of both upper and bottom leaves were higher in Virginia than in Burley varieties. The quasi-constant level was also higher in Virginia than in Burley varieties both in leaves of different positions. These results suggest that a shorter cultivation period (13-14 weeks) is more favourable for tobacco plants as protein source than for tobacco cultivated for industrial use (16-17 weeks).

3.1.2 Biochemical studies on tobacco leaves of own cultivation during curing

There are two different types of drying regarding the applied curing technology: 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. Flue-curing is primarily controlled by the air temperature and the amount of dry air that passes through the mass of tobacco.

A new curing system that was a special combination of open-air-curing and flue-curing methods was developed. Originally this combined curing method was planned for Virginia varieties of high sugar content, but was also used for Burley varieties. Later on natural curing for both tobacco types was also used. No significant differences were found in the results of natural and combined curings.
The continuous increase of peroxidase 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).
In the curing period the changes suggested a combination of a biochemical hydrolysis followed by an oxidation process. In the concentration of the water soluble polyphenol content the slight increase for both Virginia and Burley varieties was attributed to the enzymatic hydrolysis deliberating of 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 the water soluble polyphenol content and the decreasing polyphenol oxidase activity data. Polyphenol oxidase activity was higher in Burley than in Virginia varieties. This fact suggests that the role of polyphenol oxidase in the oxidation of phenolic compounds in curing is reduced compared to their direct oxidation by oxygen.
A slight increase was found in the soluble protein concentrations for both Virginia and Burley varieties in the curing period. This increase could be attributed to the partial hydrolysis of insoluble protein fraction. We found higher soluble protein concentrations in Virginia than in Burley. The end of curing period was found as the most favourable term for protein isolation from different tobacco cultivars.

3.1.3 Biochemical studies on tobacco leaves of own cultivation during fermentation

The second stage of tobacco leaf processing is the fermentation. The aim of fermentation of dried tobacco is that the processed material is ready to be stored without damage; changes should go through its taste, colour and aroma so that it makes a good raw material for further manufacturing. In one side the tissue enzymes of tobacco and on the other side the settled microbes could take part in this process.
No significant differences were found in the results of fermentation after natural and combined curing of tobacco leaves of own cultivation. In fermentation period the deactivation of the enzymes (peroxidase and polyphenol oxidase) 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.

3.2 Study of the tobacco leaves of the industrial cultivation during fermentation

In the industrial cultivation Virginia-type tobacco was different from the Burley tobacco not only in their curing, but also in their cultivation, and their physical and chemical characteristics. The flue-curing of Virginia leaves contained different phases among them yellowing and different kinds of drying treatments.

3.2.1 Biochemical studies on tobacco leaves of industrial cultivation during fermentation

The increase of peroxidase activity at the start of the fermentation was attributed to the heat sensitivity of the enzyme. The sensitivity was higher in Virginia than in Burley. During fermentation a significant decrease in enzyme activity was found. The decrease in polyphenol oxidase activity and in the concentration of both total soluble phenolic compounds and soluble protein content (both were higher were in Virginia than in Burley) were similar to the results of own cultivation.

3.2.2 Chemical analysis of tobacco leaves of industrial cultivation during fermentation

The tobacco leaves of industrial cultivation were examined not only by biochemical methods but also by instrumental analysis. Total nitrogen, protein nitrogen, total sugar, reductive sugar, alkaloid, nitrate ion and chloride ion contents were measured, but it was focused only on the different kinds of sugar contents.

No significant differences were found in the sugar contents in flue-cured Virginia compared to the results of own cultivation (open-air-cured and combined-cured). It means that the high sugar content was not so sensitive to a long curing than it was earlier supposed.

3.3 Microbiological study of the tobacco leaves during fermentation

Comparative studies of the microbiological changes were accomplished under the fermentation of Virginia (combined -cured and open-air-cured in the case of own cultivation and flue-cured in the case of industrial cultivation) and Burley (combined -cured and open-air-cured in the case of own cultivation and open-air-cured in the case of industrial cultivation). Because of the different properties of the tobacco leaves (Virginia tobacco leaves have higher sugar and lower protein content than Burley leaves) different results were expected. These examinations were measured at the Department of Microbiology and Biotechnology for the micro organisms: mesophile aerobic

microbes (among them sporeforming bacteria, and fungi – moulds and yeasts); and mesophile anaerobic microbes. In the case of moulds a determination of species was also done.

3.3.1 Microbiological studies on tobacco leaves of own cultivation during fermentation

I found interesting that far less (practically no) moulds were detected in Virginia (rich in sugar) compared to Burney (low sugar content). A far more significant increasing of mesophile aerobic microbes during fermentation was found in Virginia than in Burley. This fact was attributed to the high sugar content, therefore the high microbiological infection of Virginia during the open-air-curing or combined-curing before the fermentation. It seemed that the high sugar content of Virginia was a better substrate for mesophile aerobic microbes than the high protein content of Burley.

3.3.2 Microbiological studies on tobacco leaves of industrial cultivation during fermentation

During the microbiological studies on tobacco leaves of industrial cultivation a determination of species was also done in the case of moulds. For the measuring of mesophile aerobic microbes the technique of colony-forming unit (cfu) was used. The cfu of mesophile aerobic microbes was about four-fivefold higher on the Burley leaves before redrying treatment than on the Virginia leaves. After the redrying there was a slight increasing in count of microbes that was caused by the increasing number of aerobic sporeforming bacteria. During fermentation the aerobic microbes were pressed back, especially on the Burley's leaves. The aerobic sporeforming bacteria were slightly activated by redrying effects but after the fermentation the cfu was decreased on both tobaccos. It was considerable in case of Burley. Before redrying the cfu of yeasts was higher on Burley leaves than on Virginia leaves. During the redrying process this value was decreased, but after this process yeast's cfu was increased on the Burley strips during fermentation. The changing in cfu of moulds was very characteristic. This cfu was increased tenfold in the Virginia samples. In contrast of Virginia type tobacco, on the Burley leaves the redrying treatment and fermentation caused a reduction.

The most occurring moulds (identified by species) are demonstrated according to processing and tobacco type. The lamina of Virginia leaves was contaminated by *Aspergillus parasiticus, Aspergillus niger, Penicillium echinulatum* and *Rhizopus* species. After the fermentation two species, *Aspergillus parasiticus* and *Penicillium echinulatum* were dominant, *Rhizopus sp.* and *Aspergillus niger* were eliminated. *Aspergillus parasiticus* and *Aspergillus niger were* occured before redrying treatment on the Burley leaves but they were not isolated from the tobacco leaves after the fermentation.

It seemed that the leaves of both Virginia and Burley are good substrates of microbes. Therefore the infection of the leaves before fermentation is one of the most important factors. The high sugar content, therefore the high microbiological infection of Virginia was found during the open-air-curing or combined-curing before the fermentation. Therefore the results of industrial cultivation and own cultivation suggest an advantage of flu-curing compared to open-air-curing or combined-curing in the case of Virginia tobacco leaves.

4. NEW SCIENTIFIC ACHIEVEMENTS

1. Practically I was the first who measured the activity of so-called stress enzymes (peroxidase, polyphenol oxidase and lipoxygenase), the level of the water soluble polyphenol content (characteristic for the quality of tobacco) and the level of the water soluble protein content (characteristic for the possible use of tobacco for food industry) in eight varieties of Virginia and Burley cultivars during my own cultivation.
2. By statistic methods in the activity of peroxidase, polyphenol oxidase, the level of the water soluble polyphenol content and the level of the water soluble protein content of tobacco leaves I found significant differences according to their different positions (their age). At the same time I found that tobacco lipoxygenase cannot be used to find a convergence between the lipoxygenase isoenzyme compositions of different varieties of tobacco.
3. I found that a shorter cultivation period (13-14 weeks) is more favourable for tobacco plants as protein source than for tobacco cultivated for industrial use (16-17 weeks).
4. I found slightly increasing protein concentrations for both Virginia and Burley varieties in the curing period of both open-air-curing and combined curing. The end of curing period was found as the most favourable term for protein isolation from different tobacco cultivars.
5. A new and cheap curing system (named combined-curing) was worked out that was a special combination of open-air-curing and flue-curing methods especially for Virginia varieties of high sugar content.
6. On the basis of the comparative biochemical and microbiological studies of the effect of open-air-curing and own combined-curing methods on the fermentation period of tobacco leaves I found that the flue-curing was more advantageous for Virginia of high sugar content because during these longer curing methods the infection of Virginia by different microbes was higher than in the case of flue-curing.
7. During the biochemical and microbiological studies on the fermentation period of industrial cultivations of tobacco plants I worked out better parameters for flue-curing and

fermentation period than the presently used parameters. As the leaves of both Virginia and Burley are good substrates of microbes, the possibility of the infection before the fermentation could be an important parameter. Before and during fermentation and further processes a control of humidity (stronger than it is nowadays) is suggested.

5. CONCLUSIONS AND SUGGESTIONS

1. The possibility of different utilizations of the tobacco plant was considered because tobacco plant can be cultivated easily and economically. These utilizations could be in connection or parallel with tobacco industry. By the following of the changes of protein content during the cultivation and curing of different types of tobacco plant I made suggestions for the use of tobacco plant as protein source.
2. In my opinion the combined-curing method of tobacco leaves could be useful for further investigations.
3. On the basis of my results in connection with the biochemical and microbiological studies on the fermentation period of industrial cultivations of tobacco plant I made suggestions for the regulation of different parameters (especially humidity) during the industrial primary treatment of tobacco plant.

6. PUBLICATIONS RELATED TO THE DISSERTATION

Publications in international journals

1. Szedljak, I., Szántainé Kőhegyi, K., Kosáry, J. (2007): Preliminary biochemical studies on a model growing of different tobacco plant (*Nicotiana tabacum* L.) cultivars. J. Int. Horticult. Sci. **13**, 83-87. (if 0). ISSN 1585-0404.
2. Szedljak I., Szántainé Kőhegyi K., Kosáry J. (2010): Biochemical studies on curing and fermentation processing periods of different tobacco plant (*Nicotiana tabacum L.*) cultivars. Betrage zur Tabakforschung International/Contributions to Tobacco Res. 24, 24-28. (if 0). ISSN 1612-9237.
3. Szedljak, I., Szántainé Kőhegyi, K., Kosáry, J. (2010): Study of tobacco plant as a possible nutritive protein source. Acta Alimentaria **39**, 138-145. (if 0. 0.510). DOI: 10.1556/AAlim.39.2010.2.6.

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Short proceedings in home conferences

2. Szedljak I., Juhászné R. M., Szántainé Kőhegyi K. (2004): Dohányok mikrobiológiai jellemzőinek vizsgálata fermentálás és tárolás során. A Magyar Mikrobiológiai Társaság Évi Nagygyűlése és a X. Fermentációs Kollokvium előadás kivonatok, Keszthely, 116.
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8. Szedljak I., Juhászné Román M., Szántainé Kőhegyi K.,Tar Zs. (2005): Különböző termesztési évből származó Burley dohányminták mikrobiológiai jellemzői a fermentáció során. Microbiological Features of Burley Tobacco Grown in Different Years, Lippay -Ormos -Vas Tudományos Ülésszak Budapest, 174-175. ISBN 963-503-342-7
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