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INFLUENCE OF LIGNIN EXTRACT ON THE ACTIVITY OF ENZYMES, RELATED TO CARBOHYDRATE AND PEPTIDE DIGESTION

In cooperation with the Latvian Institute of Wood Chemistry, the following research work shows quantitative experiments to investigate the effects of different concentrations of Lignin on the activity of the enzymes Pepsin and α – Amylase. All experiments are made "in vitro" and corresponding environments were simulated. The long – term aim of this research project is, to give evidence about the different effects of lignin on human enzymes to establish a cost – efficiently, natural drug.

This research work is related to the European COST program, action CM0804:

Chemical Biology with Natural Products:

"The main objective of the Action is to advance the use of natural products as tools for chemical biology. Applying modern techniques and advancing them, natural products will prove to be instrumental in discovering target proteins and biological pathways that are of relevance to diseases. This in turn, should facilitate and speed up subsequent drug discovery efforts in the pharmaceutical industry".

Fundamentals

Lignin is a complex dendritic network polymer of phenyl propene basic units is one of the most important substances in the secondary cell wall of plant cells. It is responsible for the strength and density of wood. Next to cellulose it is the second most abundant renewable carbon source on earth. Lignin is unusual in comparison with other biopolymers because of its heterogeneity and lack of a defined primary structure. Its natural function is the mechanical support through strengthening of wooden proportions in trees. Also antioxidant properties of Lignin were clearly documented. It is difficult to use lignin in its natural form in any analytical methodology therefore the lignin used in this research has been fractioned with Isopropanol.

Depending on the simulated medium, either amylase and starch (oral cavity) or pepsin and casein (stomach) were used as substrates and target enzymes.

Pepsin is an endopeptidase resident in the gastric cavity. It is secreted as inactive zymogene called Pepsinogen and activated by autocatalysis. Autocatalysis is triggered by an acidic stomach medium caused by the secretion of HCL during the cephalic and gastric stages of digestion. Pepsin catalyzes the hydrolysis of peptide bonds adjacent to aromatic and branched-chain amino acids and methionine.

Amylases are enzymes, produced in the human body by salivary glands and the pancreas, that catalyzes the breakdown of starch into monosaccharides. Salivary α(1-4)-amylase hydrolyses randomly α(1-4) glucoside bonds of polysaccharides in the oral cavity. The resulting products of this reaction are dextrins and a mixture of glucose, maltose, maltotriose and small branched dextrins. The quantitative composition of this mixture depends on different factors: concentrations of substrates, concentration of amylase, time, temperature, pH, the concentrations of activators and inhibitors. The optimal conditions for salivary amylase are a pH between 6,8 and 7,2 and a temperature between 37° and 38°C. The amylase activity is characterized with amyloclastic force, that is the volume of the 0,1% starch solution in milliliters that is hydrolysed by 1ml of saliva at 38°C dyring 30 minutes. The amyloclastic force is denoted as $D_{30/38°}$. The average range of amyloclastic force in healthy people is from 320 to 1280.

Description of the experiments on amylase activity

For the experimental determination of the influences of Lignin on amylase activity three sets of testtubes are used, the saliva is donated by a sober student (without nutrition, chewing gum, nicotin etc.) to get clean results. All sets consists of a test and a control row. Within the first set the natural activity of amylase of the used saliva is investigated, in the second set one drop of Lignin JP II (concentration c = 1600 mg/L) is added, in the third set one drop of Lignin JP I (concentration c = 800 mg/L), in the fourth set one drop of diluted Lignin JP I (concentration c = 400 mg/L) and in the fifth set one drop of diluted Lignin JP I (concentration c = 200 mg/L). The Lignin, that is used in the experiments is extracted from black alder trees.

First test row without Lignin

Testtube number	Saliva quantity in ml	Colour
1-4	1/20-1/160	yellow
5	**1/320**	**reddish-brown**
6-10	1/640–1/10240	blue

First control row without Lignin:

Testtube number	Saliva quantity in ml	Colour
1-4	1/20 – 1/160	yellow
5	**1/320**	**reddish-brown**
6-10	1/640	blue

Calculation of the native amyloclastic force of the donated saliva after 30 minutes at 38°C: Testtube number 5 showed a reddish-brown colour after 30 minutes at 38°C and 1 drop of 1% iodine solution, the saliva quantity in it is 1/160 ml. This number is used for the calculation of the amyloclastic force. $D_{30/38°}$ = (1*2)/(1/320) = **640.**

Second test row with 1 drop of Lignin II (c = 1600 mg/L) in each tube (see second picture in appendix):

Testtube number	Saliva quantity in ml	Colour
1-7	1/20–1/1280	yellow
8	**1/2560**	**reddish-brown**
9-10	1/5120–1/10240	blue

Second control row with 1 drop of Lignin II (c = 1600 mg/L) in each tube:

Testtube number	Saliva quantity in ml	Colour
1-7	1/20–1/1280	yellow
8	**1/2560**	**reddish-brown**
9-10	1/5120–1/10240	blue

Calculation of the amyloclastic force of the donated saliva with 1 drop of Lignin II after 30 minutes at 38°C: Testtube number 8 showed a reddish-brown colour after 30 minutes at 38°C and 1 drop of 1% iodine solution, the saliva quantity in it is 1/2560 ml. This number is used for the calculation of the amyloclastic force. $D_{30/38°}$ = (1*2)/(1/2560) = **5120.**

Third test row with 1 drop of Lignin I (c = 800 mg/L) in each tube (see third picture in appendix):

Testtube number	Saliva quantity in ml	Colour
1 - 6	1/20 – 1/640	yellow
7	**1/1280**	**reddish-brown**
8 - 10	1/2560 – 1/10240	blue

Third control row with 1 drop of Lignin I (c = 800 mg/L) in each tube:

Testtube number	Saliva quantity in ml	Colour
1 - 6	1/20 – 1/640	yellow
7	**1/1280**	**reddish-brown**
8 - 10	1/2560 – 1/10240	blue

Calculation of the amyloclastic force of the donated saliva with 1 drop of Lignin I after 30 minutes at 38°C: Testtube number 7 showed a reddish-brown colour after 30 minutes at 38°C and 1 drop of 1% iodine solution, the saliva quantity in it is 1/1280 ml. This number is used for the calculation of the amyloclastic force. $D_{30/38°}$ = (1*2)/(1/1280) = **2560.**

Fourth test row with 1 drop of diluted Lignin I (c = 400 mg/L) in each tube (see fourth picture in appendix):

Testtube number	Saliva quantity in ml	Colour
1 - 6	1/20 – 1/640	yellow
7	**1/1280**	**reddish-brown**
8 - 10	1/2560 – 1/10240	blue

Fourth control row with 1 drop of diluted Lignin I (c = 400 mg/L) in each tube:

Testtube number	Saliva quantity in ml	Colour
1 - 6	1/20 – 1/640	yellow
7	**1/1280**	**reddish-brown**
8 - 10	1/2560	purple, blue

Calculation of the amyloclastic force of the donated saliva with 1 drop of diluted Lignin I (c =400mg/L) after 30 minutes at 38°C: Testtube number 7 showed a reddish-brown colour after 30 minutes at 38°C and 1 drop of 1% iodine solution, the saliva quantity in it is 1/1280 ml. This number is used for the calculation of the amyloclastic force. $D_{30/38°}$ = (1*2)/(1/1280) = **2560.**

Fifth test row with 1 drop of diluted Lignin I (c = 200 mg/L) in each tube (see fifth picture in appendix):

Testtube number	Saliva quantity in ml	Colour
1-7	1/20–1/1280	yellow
8	**1/2560**	**reddish-brown**
9-10	1/5120–1/10240	blue

Fifth control row with 1 drop of diluted Lignin I (c = 200 mg/L) in each tube:

Testtube number	Saliva quantity in ml	Colour
1-7	1/20–1/1280	yellow
8	**1/2560**	**reddish-brown**
9-10	1/5120–1/10240	blue

Calculation of the amyloclastic force of the donated saliva with 1 drop of diluted Lignin I (c = 200mg/L) after 30 minutes at 38°C: Testtube number 8 showed a reddish-brown colour after 30 minutes at 38°C and 1 drop of 1% iodine solution, the saliva quantity in it is 1/2560 ml. This number is used for the calculation of the amyloclastic force. $D_{30/38°} = (1*2)/(1/2560) =$ **5120.**

Description of the experiments on pepsin activity

For the experimental determination of the influence of Lignin on the activity on the enzyme Pepsin, six sets of test tubes are used. Casein, is used as a protein source in equal amounts of 1 g in each test row. Every set consists of a test and a control row. Lignin is used in five groups in two different concentrations. JP-3 (concentration c = 800 mg / L) is added to the Sets 2 – 4, JP-4 (concentration c = 1.600 mg / L) is used in the sets 5 and 6. The first part of the experiment is the simulation of a acidic gastric medium (HCL is added) to show the effects of Pepsin on the Casein resin and, by a visual observation, determine the changes of Casein in the different test rows. In the second part, qualitative verification of the pepsin activity is given by the biurete reaction, a chemical test for detecting the presence of proteins (violet colour) or pink (light violet) in the presence of short-chain polypeptides (evidence on Pepsin activity).

For visual observation:

Set	Tube I	Tube II
Control Group I *no Lignin*	swelling	swelling
Control Group II *1 drop (1 / 20 ml) of JP-3*	**highest swelling**	**highest swelling**
Experiment I *1,0 ml JP-3*	minor swelling	minor swelling
Experiment II *0,5 ml JP-3*	intermediate swelling	intermediate swelling
Experiment III *1,0 ml JP-4*	partially soluted	partially soluted
Experiment IV *0,5 ml JP-4*	soluted	soluted

Control Group II showed, that the greatest effects of lignin on pepsin are present in low amounts. Higher amounts (for JP-3) and concentrations (for JP-4) seemed to act as an inhibiting factor on the enzyme activity.

For biurete reaction:

Set	Tube I	Tube II
Control Group I *no Lignin*	(b): blue sediment (a): light violet colour	(b): blue sediment (a): light violet colour
Control Group II *1 drop (1 / 20 ml) of JP-3*	(b): blue sediment (a): pink (light violet) colour	(b): blue sediment (a): pink (light violet) colour
Experiment I *1,0 ml JP-3*	(b): blue sediment (a): dark violet – brownish colour	(b): blue sediment (a): dark violet – brownish colour
Experiment II *0,5 ml JP-3*	(b): blue sediment (a): dark violet – brownish colour	(b): blue sediment (a): dark violet – brownish colour
Experiment III *1,0 ml JP-4*	(b): blue sediment (a): dark violet colour	(b): blue sediment (a): dark violet colour
Experiment IV *0,5 ml JP-4*	(b): blue sediment (a): dark violet colour	(b): blue sediment (a): dark violet colour

The biurete reaction has proved the results from the visual observation. As stated in the theory, the colour reflects the presence of polypeptide bonds. Depending on the intensity, the test gives qualitiativ verification of proteins (violet – dark violet) or short-chain polypeptides if the colour is pink (or light violet).

Conclusions

A strong influence of Lignin on amylase activity can be concluded from the observations, that were made during the first experiment. Even in very low concentrations the amyloclastic force increases (factor 2– 8) clearly, there is no linear dependency between the used concentrations and amyloclastic force.

As seen in the second experiment, different effects of Lignin on Pepsin are present. Interestingly, the higher the amounts and concentrations, the lower the effects are, thus, in these regions, an inhibiting role of Lignin is assumed.

The exact biochemical mechanisms between the interaction of Lignin with amylase, pepsin and other human enzymes is supposed to be content of our continuing research.

Expectations for the futures may be the use of lignin as a natural compound in drugs. Also from the economical point of view Lignin is quite interesting, due to its cheap extraction from renewable sources.

References:

1. http://www.cost.eu/domains_actions/cmst/Actions/CM0804