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INVESTIGATIONS TOWARDS THE BINDING MECHANISMS OF VEGETABLE TANNING AGENTS TO COLLAGEN

INTRODUCTION

Plants contain secondary metabolites to fend off predators. The substances react with proteins like viral proteins or proteins of taste buds of mammals and this leads to the loss of biological function. These properties can also be used to cross link collagen to enhance thermal and mechanical stability and resistance against enzymatic degradation.

The classical vegetable cross linkers are classified into two chemically different groups: hydrolysable and condensed tanning agents. The interactions of hydrolysable agents are based on hydrogen bonds and electrostatic forces. Condensed tannins react via hydrogen -and covalent bonding which are not stable against acids.

Approximately since 15 years, plant metabolites of a third group are established as cross linkers for collagen materials, named Iridoids and Secoiridoids. Most famous are Oleuropein from olive leaves and Genipin in gardenia fruits. These substances are often glycosilated and have to be activated by enzymatic or acidic deglycosilation.

The principle reaction mechanism of Iridoid/Secoiridoid-linking to proteins is proposed as ring opening after deglycosilation and Schiff-Bas-formation with the aldehyde- groups [1].

The aim of the study was to screen extracts of plants containing this kind of substances for their covalent cross linking activity towards collagen.

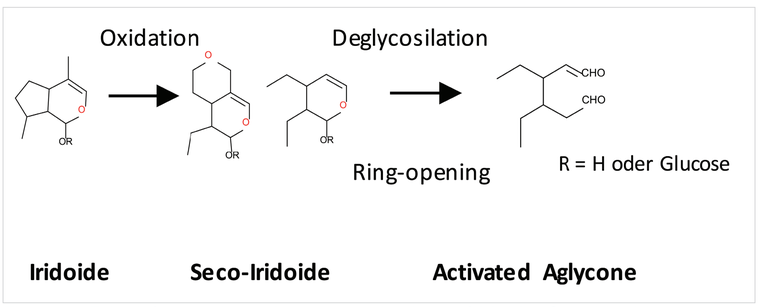

Figure 1: Principle reaction scheme of activation of Iridoids /Secoiridoids

METHODS

EXTRACTION:

- Plant materials were collected or bought from pharmacy
- Plants were extracted with water and water/ethanol 1:1 with a defined volume to mass ratios at 50°C, 5 h, three times.
- Extracts were concentrated with rotation evaporator and lyophilized

CROSS LINKING

- Hide powder was incubated with solutions of dry extracts or key substances with defined and constant concentrations in buffer at pH 7 for 5 h at 23 °C
- Deglycosilation was done with ß-Glucosidase from almond
- The cross linked hide powder was washed and dried

IDENTIFICATION OF CROSS LINKING ACTIVITY[3]

Bound amine groups (ASA):

- Samples were hydrolyzed at 110°C with 6 N HCl
- Amino acid composition was determind with amino acid analysator (Biochrom 30++) (ASA)
- The decrease of Lysine peak area compared with non-crosslinked hide Powder was set as percentage of bound Lysine groups

IDENTIFICATION OF IRIDOID/ SECOIRIDOID KEY SUBSTANCES[2]

- Aqeous solution of extracts were analyzed with HPLC (Shimadzu, stationary phase C18 –column (Supelco), mobile-phase: Acetonirile-Water-gradient, detection: Diode array –Detector (DAD)
- Quantification and Identification with reference substances (Phytolab, Sigma-Aldrich, Wako, Extrasynthese)

RESULTS

Twenty plants from the order of Lamiales and Gentianales were extracted and analyzed (Table 1).

Preliminary cross linking experiments with selected key substances with the chemical structure of Iridoids and Secoiridoids and hide powder with and without activation by ß-Glucosidase were done.

Key substances with cross linking activity were quantified with HPLC in aqueous and aqueous-ethanolic extracts. (Table 1). The contents differ considerably between both extracts. This is caused on one hand by different polarity of substances on the other hand by different activities of plant own enzymes for deglycosilation. Especially, in the aqueous solutions of plants from Oleaceae and Gentianeace family seems to be more deglycolisated than in aqueous-ethanolic extracts. In the Gentiana and Centaury extracts the aglycons of Sweroside, Swertiamarin and Gentiopicroside can be easyly identified (Figure 2). Unfortunately the identification of the Oleuropein aglycon is not possible so far as well as the aglycons of Aucubin and Catalpol.

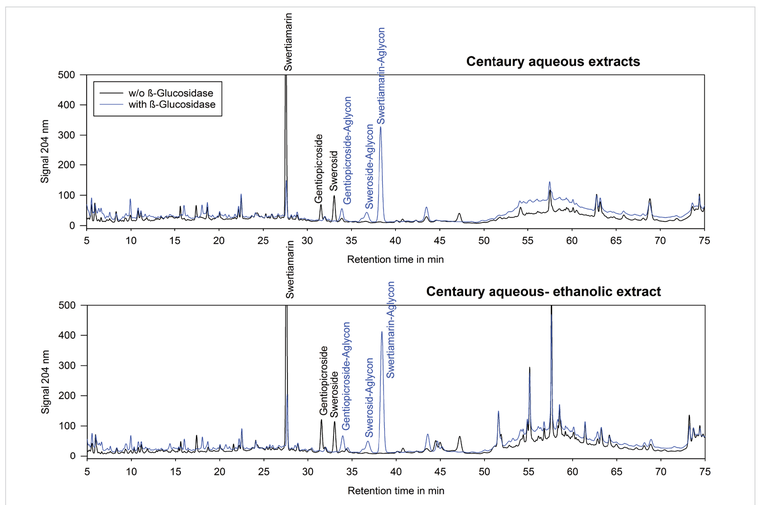

Figure 2: Chromatograms of aqueous and aqueous-ethanolic extracts of Centaury with and without ß-Glucosidase

Figure 3 shows the percentage of covalent bound Lysine groups in hide powder, treated with aqueous extracts of different plants. The plants of the plant family Oleaceae show the highest cross linking activity, followed by the two plants from the family of Gentianaceae. The plants of other families containing Aucubin and Catalpol show only weak or no cross linking activity. The application of the ASA–methode to detect cross-linked Lysine-groups exclude all the weak noncovalent interactions and covalent but non-acid-stable cross-links.

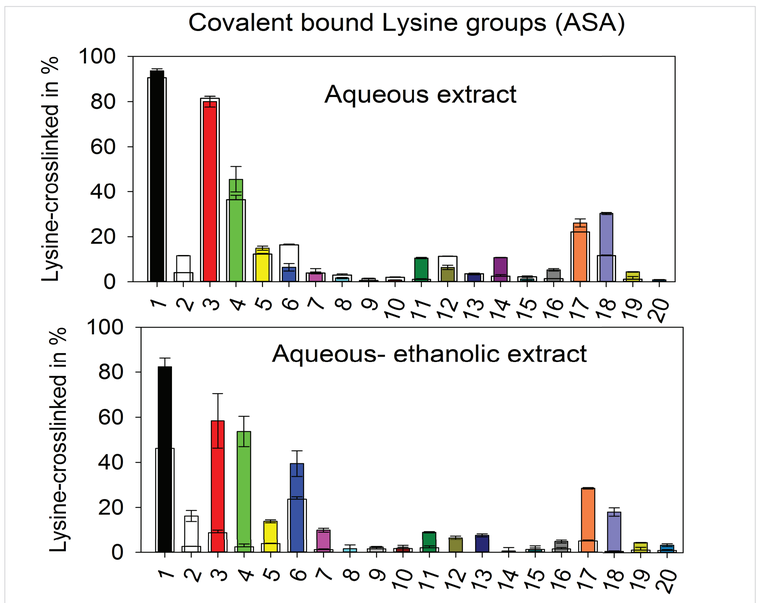

Figure 3: Covalent bound Lysin-groups (ASA) for hide powder, treatet with aqueous and aqueous-ethanolic extracts of different plants (coloured bars: with ß-Glucosidase, transparent bars without ß-Glucosidase

Table 2: Investigated plants and the content of key substances with corresponding cross linking activity after aqueous and aqueous–ethanolic extraction

Plant family	No.	Plant	Key substance	Matter content in % (mass of dry extract)*	
				Extraction with water-ethanole 1:1	Extraction with water
Oleaceae	1	Privet leaf *(Ligustrum vulgare)*	Oleuropein	4,1	0
Oleaceae	2	Privet stem *(Ligustrum vulgare)*	Oleuropein	7,6	0
Oleaceae	3	Olive leaf *(Olea europea)*	Oleuropein	10,9	1,0
Oleaceae	4	Lilac leaf *(Syringa vulgaris)*	Oleuropein	6,4	0,2
Oleaceae	5	Lilac stem *(Syringa vulgaris)*	Oleuropein	4,7	0,3
Oleaceae	6	Ash bark *(Fraxinus excelsior)*	Oleuropein	6,2	0
Oleaceae	7	Ash leaf *(Fraxinus excelsior)*	Oleuropein	1,1	0
Oleaceae	8	Forsythia leaf *(Forsythia intermedia)*	Forsythid	/**	/**
Oleaceae	9	Forsythia stem *(Forsythia intermedia)*	Forsythid	/**	/**
Plantaginaceae	10	Speedwell *(Veronica offiinalis)*	Catalpol Aucubin	0 0	0 0
Plantaginaceae	11	Plantain *(Plantago lanceolate)*	Catalpol Aucubin	6,5 2,0	7,0 2,2
Scrophulariaceae	12	Figwort roots *(Scrophularia nodosa)*	Catalpol Aucubin	0 0,2	0 0
Scrophulariaceae	13	Mullein *(Verbascum thapsus)*	Catalpol Aucubin	2,5 0,2	0 0
Orobanchaceae	14	Eye bright *(Euphrasia rostkoviana)*	Catalpol Aucubin	0 2,1	0 0,7
Pedaliaceae	15	Devils claw roots *(Harpagophytumproc)*	Aucubin	0	1,5
Verbenaceae	16	Chaste tree seeds *(Vitex Agnus-castus)*	Aucubin Agnusid	1,9 1,5	0,9 0,3
Gentianceae	17	Gentian roots *(Gentiana lutea)*	Gentiopicroside	5,7	1,8
Gentianceae	18	Centaury *(Centaurm erythraea)*	Gentiopicroside Sweroside	0,9 1,3	0,6 2,3
Rubiaceae	19	Yellow bedstraw *(Galium verum)*	Monotropein	0	0
Rubiaceae	20	Cleavers *(Galium aparine)*	Monotropein	0	0

The activities of the aqueous extracts are higher than the activities of aqueous-ethanolic extracts (except ash bark). The latter becomes more active after deglycosilation by adding of ß-Glucosidase wheras the aqueous extracts are less influenced by the addition of ß-Glucosidase. From this it can be concluded that in aqueous extracts plant own enzymes (Gluxosidases, Polyphenoloxidases), which induce the activation of Iridoids/ Secoiridoids, are more active than in aqueous-ethanolic extracts. This is confirmed by the comparison of the contents of the key substances (non-active aglycons) in aqueous and aqueous-ethanolic extracts.

CONCLUSION

Through screening of different plants containing Iridoids/ Secoiridoids four plants could be selected, which extracts yield very high cross linking activity. This are the extracts of privet leafs and lilac leafs in addition to the potential of olive leafs already known from the plant family of Oleaceae. Besides oleuropein the privet leaf contains more similar substances which enhance the cross linking activity compared with olive leafs. The Gentian and Centaury extract show moderate cross linking activity. The active substances seem to be the aglycons of Gentiopicroside and Sweroside.

(1) Konno, K.; Hirayama, C.; Yasui, H.; Nakamura, M. Enzymatic activation of oleuropein: a protein crosslinker used as a chemical defense in the privet tree. Proc. Natl. Acad. Sci. 1999, 96, 9159–9164.
(2) Romani, A.; Baldi, A.; Mulinacci, N.; Vincieri, F.; Tattini, M. Extraction and iden tification procedures of polyphenolic compounds and carbohydrates in Philly rea (Phillyrea angustifolia L.) leaves. Chromatographia 1996, 42, 571–577.
(3) Schröpfer, M.; Meyer, M. Investigations towards the binding mechanisms of vegetable tanning agents to collagen. Research Journal of Phytochemistry 2016

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