The Action of *Saraca asoca* Roxb. de Wilde Bark on the PGH$_2$ Synthetase Enzyme Complex of the Sheep Vesicular Gland

T. B. Middelkoop and R. P. Labadie

Sectie Farmacognosie, Rijksuniversiteit Utrecht, Catharijnesingel 60, Utrecht

Extracts of *S. asoca* bark and pure compounds isolated from the bark were tested for properties that might inhibit the conversion of arachidonic acid by the PGH$_2$ synthetase. They were assayed spectrophotometrically with adrenaline as cofactor. Methanol- and ethyl acetate extracts inhibited the conversion. The observed inhibition was confirmed in an oxygraphic assay. Two procyanidin dimers from the ethyl acetate extract showed enzyme catalyzed oxidation in our assay. The ether extract of the bark was also found to contain yet unknown substances which were capable of being oxidised by the PGH$_2$ synthetase. The combined action of the components of the bark may explain the mode of action of the drug Asoka Aristha, the main ingredient of which is the bark of *S. asoca*. The drug is traditionally used in Sri Lanka to treat menorrhagia.

Introduction

The traditional drug Asoka Aristha is used in Sri Lanka to treat menstrual disorders such as menorrhagia. The main ingredient of the drug is the bark of *Saraca asoca* Roxb. de Wilde or that of *Rhododendron arboreum* Nees. A review of the ethnopharmacology of the drug has been published [1]. Women suffering from dysfunctional menorrhagia and menorrhagia caused by the use of an IntraUterine Device showed abnormally high levels of PGE$_2$ and PGF$_{2\alpha}$ [2] in their endometrium tissue. It has been shown in double blind clinical trials that the use of PG synthesis inhibiting drugs decreased blood loss during menstruation [3]. Since the bark of *S. asoca* is one of the main ingredients in the drug Asoka Aristha we decided to test extracts and pure compounds found in this bark for their influence on the PGH$_2$ synthetase *in vitro*. In this paper we report our investigation on the question: whether inhibition of the prostaglandin synthetase could be the possible mode of action. We did so with a spectrophotometric assay in which the forming of adrenochrome from adrenaline, a cofactor in the enzyme reaction, was measured. The possibility exists that if the test substance in the assay is a reducing agent like adrenaline itself then this can be oxidised instead of adrenaline and a slowing down of the adrenochrome production is observed while the arachidonic acid conversion is not inhibited. To exclude this possibility the test substances were also measured in an oxygraphic assay if they showed inhibition in the spectroscopic assay.

Materials and Methods

The enzyme preparation

PGH$_2$ synthetase enzyme from sheep vesicular glands was prepared by partly following a procedure described by van der Ouderaa and Buytenhek [4]. The tween 20 solubilised microsomal fraction was gel chromatographed on an ACA-34 column as described [4] but in the eluent the dialyzable detergent 0.1% β-octyl glucoside was used instead of tween 20. The fractions were screened for activity and the active fractions obtained were pooled and concentrated using an Amicon cell with an XM-50 filter. The concentrated enzyme was divided into 1 ml portions and stored at −80 °C.

Spectrophotometric assay for PG synthetase activity

To test the enzyme activity we used a method described by Takeguchi and Sih [5]. In a Beckmann model 25 spectrophotometer equipped with temperature control set at 25 °C a cuvette with 2.0 ml buffer TrisHCl 0.05 M pH = 8.3 and detergent 0.05% (Tween 20), was inserted. The cuvette was warmed up for 1 min and after that 40 µl of a L-adrenaline tartrate 5% solution and 100 µl of the enzyme solution containing 2.2 g/l protein (FolinCiocalteau estimation) [6] were added. The enzyme was incubated for 2 min and then 50 µl of arachidonic acid (from Janssen Chimica, Belgium) solution (1.92% w/v in toluene, diluted 1:10 with the buffer) were added.

The same solutions were put into the reference cuvette except for the enzyme solution. The change in absorbancy at 480 nm, measured at time point zero and expressed in μmol adrenochrome/min was taken as a measure of PG synthetase activity. In this set up the enzyme had a specific activity of 2.4 units/mg protein. The relative standard deviation in this test was 5% (calculated from 4 measurements). To study the inhibiting properties of our test substances part of the 2.0 ml buffer solution was replaced with the desired amount of the test solution before the enzyme solution was added. The test solutions were prepared by mixing the test substances with the buffer solution for 20 min in an ultrasonic bath. If the substance did not dissolve completely it was filtered through paper before use. The concentration of the prepared test solution is expressed in g/l buffer and if it did not dissolve completely the suffix filtrate was added. The IC50 was calculated from at least 4 measurements by plotting the inhibition (expressed as a % of the non inhibited velocity of the reaction) against the log of the concentration. A linear relation was assumed and the regression line calculated. The 50% inhibition concentration was determined using this line.

Oxygraphic assay for prostaglandin synthetase activity

In a Gilson oxygraph equipped with a Clark oxygen electrode the oxygen consumption was studied according to the following procedure: In the measuring cell of the oxygraph thermostated at 25 °C were successively pipetted 1.6 ml of a buffer solution (TrisHCl 0.05 M pH = 8.3 and 0.05% detergent), 40 μl of a L-adrenaline tartrate 5% solution and 40 μl of an enzyme solution. The enzyme solution for the oxygraph measurements was a freezedried non-washed microsomal fraction of sheep vesicular gland (generously supplied to us by Unilever, Holland) 50 mg of the enzyme were suspended in 1 ml of the buffer. After 2 min in the cell the reaction was started by addition of 2 μl of an arachidonic acid solution (61 g/l in ethanol). To study the inhibiting properties of our test substances part of the 1.6 ml buffer solution was replaced with the desired amount of the test solutions. The test solutions were prepared as described above. The recorded change in the oxygen consumption measured at time point zero and expressed as μmol oxygen/min was taken as a measure of PG synthetase activity. The IC50 was calculated as above.

Preparation of the extracts

Soxhlet extracts: 100 grams of powdered bark of *S. asoca* were extracted in a Soxhlet apparatus successively with:

petroleum
ether 40−60 °C   for 12 hours, yielding   0.62 g extract

ether   for 24 hours, yielding   0.3 g extract

ethyl acetate   for 56 hours, yielding   3.1 g extract

methanol   for 50 hours, yielding 11.1 g extract

Procedure for the water extract: 3 g of powdered bark were heated over a boiling water bath for 10 min in 100 ml of a buffer solution TrisHCl 0.05 M pH = 8.3 and cooled to room temperature for 30 min in an ultrasonic bath. This mixture was filtered through paper and the filtrate was directly used as a test solution.

Pure compounds

The pure compounds procyanidin B2 and procyanidin B (11'deoxy) were isolated from the ethyl acetate extract as described in [7]. The (−)-epicatechin was a commercial product from Roth (West Germany).

Results

When we used the above mentioned spectroscopic assay method to test the extracts for properties that might inhibit the PGH$_2$ synthetase system we noticed to our surprise, that before the arachidonic acid was added some of the extracts and some of the pure compounds isolated showed an enzyme catalyzed reaction in which adrenaline was converted to adrenochrome. The properties of this reaction were more closely investigated and the results are shown under the heading substrates section: pure compounds. In Table I the results are conveniently arranged and in the following text the results are described in two parts one under the heading inhibition and one under the heading substrates.

Inhibition Extracts

Methanol extract (1 g/l)

99% of this extract consisted of a mixture of oligomeric procyanidins, the main constituent being

Table I. Survey of the results.

Test substances	Spectroscopic assay		Oxygraphic assay
	Substrate reaction[1]	IC50	IC50
Extracts			
water extract	yes	n.p.[2]	n.p.
methanol extract	no	49 mg/l	26 mg/l
ethyl acetate extract	yes	26 –	16 –
ether extract	yes	n.p.	n.p.
petroleum ether extract	no	no	–
Pure compounds			
(−)-epicatechin	no	302 μmol	–
procyanidin B2	yes	n.p.	n.p.
procyanidin B (11'dehydro)	yes	n.p.	n.p.

[1] substrate reaction means: Observation of a reaction in which adrenochrome is formed before arachidonic acid is added.
[2] n.p. means: not possible to measure inhibition because of the reaction in which adrenochrome is formed before arachidonic acid is added.

a tetramer [7]. The extract did not show a direct reaction in which adrenochrome was formed and when arachidonic acid was added inhibition was observed; the calculated IC50 was 49 mg/l. In the oxygraphic assay also an inhibition was measured. The IC50 was 26 mg/l.

Ethyl acetate extract (1 g/l)

77% of this extract consisted also of a mixture of oligomeric procyanidin, the main constituent is here also a tetramer; other identified constituents were (−)-epicatechin (2%); procyanidin dimer B2 (2.4%); procyanidin dimer B (11'deoxy) (1.2%) [7]. At a high concentration of 90 mg/l we observed a reaction, in which adrenochrome was formed, before arachidonic acid was added; the initial velocity of the reaction was 5 μmol/min. In spite of this reaction we attempted to measure the inhibition after addition of arachidonic acid. In this case the IC50 was 26 mg/l. In the oxygraphic assay also an inhibition was measured. The IC50 was 16 mg/l.

Petroleum ether extract (1 g/l, filtrate)

When arachidonic acid was added the conversion was not inhibited.

Pure compound

(−)-Epicatechin

This compound has been tested before by Wurm *et al.* with a different assay method; they found an IC50 = 120 μmol [8]. In our assay the compound did not show a reaction in which adrenochrome was formed before arachidonic acid was added, for the arachidonic acid conversion we found an IC50 = 302 μmol. In order to compare our assay with others we also measured the IC50 of indometacin and found 14 μmol (Wurm *et al.* [8] found 5.6 μmol). These results ascertained, that our assay is a suitable one for comparing the inhibiting action of various compounds. In order to find out whether the inhibition of (−)-epicatechin was a reversible one we tested this compound first by pre-incubating the enzyme for 2 min, second by pre-incubating the enzyme for 2 min in a concentration that was 6 times higher as that used in the previous experiment and thirdly no pre-incubation was used. In all three cases the inhibitions were of the same magnitude 28, 25, 28%. These results suggest that the inhibition is reversible.

Substrates Extracts

Ether extract (1 g/l, filtrate)

This extract showed a reaction in which adrenochrome was formed before arachidonic acid was added. At a concentration of 100 mg/l the initial velocity of this reaction was 100 μmol/min.

Water extract (13.6 g/l, filtrate)

The water extract in the assay showed, before arachidonic acid was added, a reaction in which adrenochrome was formed. The initial velocity was 11 μmol/min and after 8 min 37 μmol adrenochrome had been formed.

Petroleum ether extract (1 g/l, filtrate)

This extract did not show a reaction in which adrenochrome was formed.

Pure compounds

Procyanidin B2

This compound is a dimer consisting of 2 (−)-epicatechin molecules covalently bonded between C4 of the upper and C8 of the lower flavanol unit. This

substance showed in the assay a reaction in which adrenochrome is formed before arachidonic acid was added. 126 μmol of the compound formed 22 μmol adrenochrome/min. This reaction was not inhibited by indometacin when the latter was added in a concentration that would have caused 70% inhibition of the arachidonic acid conversion. In order to find if oxygen was consumed in the reaction the oxygen was removed from the reaction mixture by bubbling with nitrogen for 15 min. This caused a considerable (50%) decrease in the reaction velocity. A try-out with an oxygraph showed that PGH_2 synthetase and procyanidin B2 in buffer without addition of adrenaline also consumed oxygen. We concluded that adrenaline is not a prerequisite for the occurrence of the reaction. However as in the case of the arachidonic acid conversion the reaction rate was enhanced when the reaction mixture contained adrenaline.

Procyanidin B (11′deoxy)

This compound is a dimer consisting of an epicatechin in the upper and an afzelechin in the lower half of the molecule covalently bonded between C4 and C8 as in procyanidin B2. This substance also showed in the assay a reaction in which adrenaline was converted to adrenochrome before arachidonic acid was added. Oxygen was also consumed in this reaction and the reaction was not inhibited by indometacin. The arachidonic acid conversion inhibitor (−)-epicatechin however was found to inhibit more than 90% of the conversion of procyanidin B (11′deoxy) in a concentration that would inhibit 90% of the conversion of arachidonic acid. The above mentioned compounds are readily oxidised in a non enzymatic reaction and in order to prove that the conversions we measured are really enzymatic we tested the compound procyanidin B (11′deoxy) at a constant concentration (189 μmol) and varying enzyme concentrations (21−102 mg/l). A plot of reaction velocity against 5 enzyme concentra-

tions yielded a straight line (correlation coefficient = 0.995) and at zero enzyme concentration the reaction velocity was within the narrow limit of the standard deviation. We concluded that the reaction is completely enzyme catalyzed in a linearly concentration dependent way.

Discussion

From our experiments we conclude that certain components in the bark of *S. asoca* have an influence on the PG-synthetase enzyme. The compound (−)-epicatechin is not the only component causing inhibition. The higher flavanol oligomers are also able to inhibit the arachidonic acid conversion. This inhibition was shown in the spectroscopic as well as in the oxygraphic assay; leaving no doubt that the arachidonic acid conversion is inhibited. The dimers procyanidin B2 and procyanidin B (11′deoxy) can act as substrates for the cyclooxygenase enzyme; oxygen is a prerequisite for this reaction. These compounds like their monomer analogue (−)-epicatechin can be readily oxidised in a non enzymatic reaction. In our view the reactions we observed are specific. An analysis of the product(s) formed may elucidate the specificity of this conversion. The ether extract also contained components that could act as a substrate for the PGH_2 synthetase enzyme. The fact that the ether extract did not contain procyanidins suggests that more compounds are able to serve as substrate. The properties of these compounds are worth investigating. In any case it is very likely that during the conversion of these compounds the PGH_2 synthetase is destroyed by oxygen radicals as also occurs in the arachidonic acid conversion [9]. In this way the availability of the enzyme for PG synthesis is diminished which contributes to a lower level of PG's in the tissue. We conclude that both mechanisms, the inhibition of PG synthesis and the destruction of the PG synthetase, can be an explanation for the use of the indigenous drug Asoka Aristha in the treatment of menorrhagia.

[1] T. B. Middelkoop and R. P. Labadie, J. Ethnopharmacology **8**, 313−320 (1983).
[2] Eve A. Willman, W. P. Collins, and S. G. Clayton, Brit. J. Obst. Gyn. **83** (3), 337−341 (1976).
[3] H. R. Lindner *et al.*, Advances in PG and Thromboxane Res. **8**, 1385−1387 (1980).
[4] F. J. G. van der Ouderaa and M. Buytenhek, Meth., Enzym. **86**, 60−68 (1982).
[5] Clyde Takeguchi and Charles J. Sih, Prostaglandins **2** (3), 169−184 (1972).
[6] J. Leggett Barley, Elsevier Publishing Company 1967.
[7] T. B. Middelkoop and R. P. Labadie, Z. Naturforsch. **40b**, 855−857 (1985).
[8] J. Baumann, F. v. Bruchhausen, and G. Wurm, Prostaglandins **20** (4), 627−639 (1980).
[9] L. J. Marnett, P. Wlodawer, and B. Samuelsson, Biochem. Biophys. Res. Commun. **60**, 1286 (1974).