Anticomplement Activity of Lysine Complexes of Propolis Phenolic Constituents and Their Synthetic Analogs

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Propolis, Phenoic Constituents, Anticomplement Activity

Several phenolic constituents of propolis and their synthetic analogs were derivatized with L-lysine. The ability of these complexes to alter complement activity was estimated *in vitro* in human serum. The influence of selected complexes on C3 hemolytic activity via classical pathway (CP) and alternative pathway (AP) and on zymosan-induced AP activation was determined. The results suppose that the anticomplement effect of the complexes might be related to the interaction with C3 complement component.

Introduction

A number of natural and synthetic substances have been reported to influence the complement system, which ensures the nonspecific protection of host against various infections. Propolis, a bee product, containing more than 200 individual constituents (Greenaway *et al.,* 1991; Garcia-Viguera *et al.,* 1993) has been used in folk medicine for centuries. The phenolics represent about 50% of propolis composition (Bankova *et al.*, 1982: Tomas-Barberan *et al.*, 1993; Walker and Crane, 1987; Volpert and Elstner, 1994) and among them with proved biological activity are pinocembrin, galangine, cinnamic acid, caffeic acid and its esters (Ghisalberi, 1979: Grunberger *et al.*, 1988; Ikeno *et al.*, 1991; Strehl *et al.*, 1994; Marcucci, 1995). Previously, we investigated a water-soluble derivative of propolis with L-lysine and proved that it possessed complement modulatory and antiinflammatory properties *in vitro* and *in vivo* (Ivanovska *et al.*, 1995; Ivanovska *et al.*, 1995). These data are in agreement with the reported data on antiinflammatory effect of propolis extracts and its flavanoids and phenolic constituents (Bauman *et al.*, 1980; Khayyal *et al.,* 1993; Pascual *et al.*, 1994). Immunomodulatory effect exhibited also complexes of cinnamic acid, obtained at different ratios with L-lysine. The anticomplement potency of these complexes depended on the lysine content and the results showed that one of the targets of their action was C3 complement component (Ivanovska *et al.*, 1993). Guided by the chemical structure and content in the total mixture flavones, phenolic acids and their esters identified in propolis were selected and derivatized with L-lysine. Lysine complexes of some synthetic ester analogs were also used in this study. The objective of the present work was to define the constituents responsible for the complement modulatory action of the whole mixture. The most effective of them can be further tested for antiinflammatory or adjuvant properties in appropriate *in vivo* models.

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Materials and Methods

Preparation of lysine complexes

The substances used in these experiments were obtained from the following sources: pinocembrin, pectolinarigenin and quercetin were isolated from propolis; caffeic acid was bought from Merck (Darmstadt, Germany); ferulic acid from T. Schuchardt (München); cinnamic and chlorogenic acid from Fluka (Buchs, Switzerland) and chrysin from Roth (Karlsruhe). Esters of phenolic acids were synthesized according to the method of Bankova (1990). Lysine complexes were prepared as described previously for cinnamic acid lysine complexes (Ivanovska *et al.*, 1993). The amount of lysine was determined by reaching full solubility of

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the complex in saline. For complement assays they were dissolved in veronal buffer and used within two days.

Complement inhibition assay

Classical pathway and AP activities were determined by the method of Klerx *et al.*, (1983). In the assay isotonic veronal buffer saline (VSB), pH 7.4, containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (VSB^{2+}, for CP) and VSB, containing 5 mM Mg^{2+} and 8 mM ethyleneglycol-bis-(2-aminoethyl)tetraacetic acid (VSB-EGTA, for AP) were used. Normal human serum (NHS) from healthy donors served as a complement source. Sensitized sheep red blood cells (sSRBC) were target cells in CP assay and uncoated rabbit erythrocytes (RaE) were target cells in the AP assay. NHS was preliminary titrated via both pathways in order to determine the dilution giving 50% hemolysis. Briefly, the inhibitory activity was determined as follows: 0.1 ml of serially diluted in VSB^{2+} lysine complexes were incubated with 0.1 ml of appropriately diluted NHS (1:40 for CP and 1:4 for AP) for 30 min at 37 °C. After that 0.05 ml of 2% suspension of sSRBC (CP) or RaE (AP) were added. After incubation at 37 °C for 60 min (CP) or 45 min (AP) the residual hemolytic activity of NHS was measured spectrophotometrically at 405 nm. The percentage of inhibition was calculated by the formula:

$$\frac{\%\text{ hemolysis of control} - \%\text{ hemolysis of sample}}{\%\text{ hemolysis of control}} \times 100.$$

Dose-response curves were drawn by plotting the inhibition against dilutions of the complexes and the concentration giving 50% inhibition of hemolysis (IC_{50}) was determined.

C3 residual activity

C3-deficient serum (R3) was obtained by incubation of fresh NHS for 30 min at 37 °C with activated zymosan (final concentration 5 mg/ml). A volume of 0.1 ml undiluted NHS was incubated with 200 μg of each complex diluted in 0.1 ml VSB for 30 min at 37 °C. The ability of NHS to restore hemolytic activity of R3 serum was measured as follows: serial dilutions of complex/NHS mixture in VSB^{2+} or in VSB-EGTA were made and 0.1 ml of 1:5 diluted R3 serum was added together with 0.05 ml of target erythrocytes.

Influence of complexes on zymosan-induced AP activation

Activated zymosan (30 min boiling in saline) was added as 1% suspension in VSB-EGTA (0.02 ml) to 0.1 ml NHS for 20 min at 37 °C. The complexes were added simultaneously with the activator at a final concentration of 200 μg/ml. After centrifugation for separation of zymosan the residual AP activity of NHS was determined. Percentage of the inhibition of complement hemolysis was calculated and compared to the samples where buffer was added instead of activator.

Results and Discussion

The complement system is the major factor in the pathogenesis of many infectious and inflammatory processes. The ability of lysine complex of propolis to affect complement activity *in vitro* and *in vivo* and the established relation with its immunomodulatory and antiinflammatory properties was the reason to choose the complement assays for estimation the immunomodulatory capacity of lysine complexes of individual propolis constituents. The results in Table I show that some of the complexes tested exhibited high inhibitory effect on CP activity. If the concentration range for IC_{50} from 12.0 to 50.0 μg/ml was taken for significant they might be arranged in the following order: benzyl-p-coumarate > benzyl caffeate, isoamyl caffeate > pectolinarigenin > cinnamic acid > phenetyl ferulate > chrysin > chlorogenic acid, benzyl-*o*-coumarate. Related to AP activity the highest inhibition was detected for chrysin, phenetyl ferulate > cinnamic acid > benzyl caffeate > benzyl-p-coumarate > benzyl ferulate. Four of the complexes (chrysin, phenetyl ferulate, benzyl caffeate and benzyl-p-coumarate) were capable to suppress strongly both, CP and AP activities. The complexes of quercetin, benzyl sinapate and benzyl 3,4-dimethoxycinnamate showed no effect via both pathways. The inhibition did not correlate with the lysine content. The complexes of benzyl caffeate and phenetyl caffeate are with equal content of lysine and belong to the same chemical group but differ greatly, especially in regard to CP activity. If the present data are compared to the

Table I. Effect of lysine derivatives on complement activity (CP and AP) in normal human serum (NHS).

Substance[a]	Classical pathway (IC_{50} μg/ml)	Alternative pathway (IC_{50} μg/ml)	Content (%)[b]	Substance: LY (weight ratio)
Flavanone				
Pinocembrin*	N.E.[c]	2000.0 ± 2.0	15–23	1:7.5
Flavones				
Chrysin*	30.0 ± 0.8	6.0 ± 0.2	3–6	1:5.7
Pectolinarigenin*	16.0 ± 0.4	N.E.	n.d.[d]	1:3.9
Quercetin*	N.E.	N.E.	<1	1:4.5
Phenolic acids and esters				
Cinnamic acid*	20.0 ± 0.5	16.0 ± 0.2	~1	1:8.5
Caffeic acid*	74.0 ± 1.5	8.0 ± 2.0	<1	1:3.1
Ferulic acid*	320.0 ± 2.2	240.0 ± 2.5	traces	1:1.6
Chlorogenic acid	40.0 ± 0.4	320.0 ± 2.2	–	1:1.0
Benzyl caffeate*	12.0 ± 0.4	21.0 ± 0.5	~1	1:6.6
Phenetyl caffeate*	N.E.	120.0 ± 0.8	1–2	1:6.6
Benzyl-p-coumarate*	10.0 ± 0.4	28.0 ± 0.4	traces	1:5.6
Benzyl ferulate*	120.0 ± 2.2	40.0 ± 1.8	traces	1:4.8
Phenetyl ferulate*	30.0 ± 0.5	6.0 ± 0.2	traces	1:4.3
Isoamyl caffeate	12.0 ± 0.2	80.0 ± 0.4	–	1:4.2
Benzyl-o-coumarate	40.0 ± 0.6	80.0 ± 0.7	–	1:4.3
Benzyl sinapate	N.E.	N.E.	–	1:6.9
Benzyl-2-hydroxy-3-methoxycinnamate	88.0 ± 0.8	N.E.	–	1:4.1
dimetoxycinnamate	N.E.	N.E.	–	1:4.7
WSD (water soluble derivative of propolis)	100.0 ± 2.0	160.0 ± 2.2	–	1:7.0

[a] Each substance was tested in triplicate at several doses for its ability to inhibit CP and AP activity in NHS. The amount af substance required to produce 50% inhibition of hemolysis (IC_{50} in μg/ml) was determined from dose-response curves. Data are mean ± S.D. from three determinations; [b] Amount (%) of natural constituents in the propolis phenol complex; [c] N.E. – not effective below 2000 μg/ml; [d] n.d. – not detected in this propolis sample; * Natural propolis constituents. The rest are their synthetic analogs.

previous once, obtained for complexes of cinnamic acid with different content of lysine, a conclusion might be drawn that lysine can influence the biological activity of complexes but an exact structure/activity relationship has not been established. The IC_{50} values for some phenolic constituents are ten times lower than the IC_{50} values for the whole mixture (Table I, water soluble derivative of propolis (WSD) v.s. cinnamic acid and benzyl-*p*-coumarate). This result at the one side points on the additive final effect of the WSD constituents and at the other side shows that the identification of the individual compounds with high activity is very perspective. Some of the complexes were examined for their effect on C3 functional activities via both pathways (Fig. 1). Chrysin enhanced C3 hemolysis via CP and diminished C3 activity via AP. Caffeic acid, benzyl caffeate and phenetyl ferulate weakly reduced classical pathway C3 activity. The alternative C3 activity was suppressed by com-

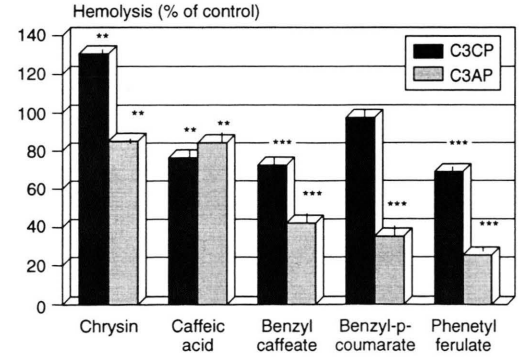

Fig. 1. Changes in C3 hemolytic activity determined via classical pathway (CP) or alternative pathway (AP) after preincubation of hunan serum (30 min, 37 °C) with complexes (200 μg/ml). Values are means ± S. D. from three determinations in three experiments. **p<0.01; ***p<0.001 as calculated to the control samples (serum + buffer) by Student's t-test.

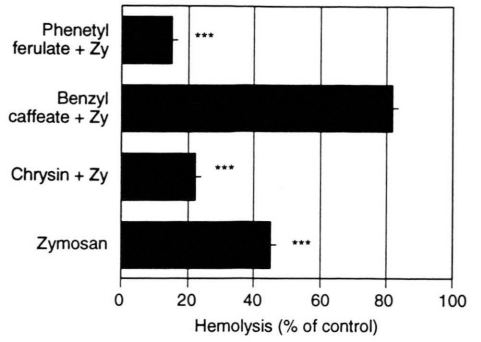

Fig. 2. Residual alternative pathway (AP) activity determined after preincubation of human serum (20 min, 37 °C) with zymosan or complexes (200 μg/ml) plus zymosan. The results are means ± S. D. from three determinations. ***$p<0.001$, Student's t-test.

plexes of benzyl-p-coumarate, phenetyl ferulate > benzyl caffeate > caffeic acid. Since the inhibitory effect of the complexes was more expressed upon total AP and alternative C3 activities, three of them were tested in zymosan-induced AP activation. Results in Fig. 2 showed that zymosan caused 50% consumption of complement activity at the concentration used. Complexes of chrysin and phenetyl ferulate significantly potentiated zymosan action, which additionally decreased the residual activity of NHS. In contrast, the complex of benzyl caffeate in combination with zymosan lowered the effect of zymosan, which resulted in an increase of hemolysis. Most of the active complexes affected definitely one of the pathways. The data confirmed the suggestion that C3 might be one of the target molecules. As a whole the inhibition of the alternative C3 activity was more expressed than that of the classical C3 activity. The application of complexes of chrysin, benzyl caffeate and phenetyl ferulate in zymosan-induced AP hemolytic assay indicated that their mode of action was not unique. The two esters, benzyl caffeate and phenetyl ferulate were with opposite influence on the process of activation. It is also possible that one complex might counteract two or more complement components.

In conclusion, some of the propolis constituents were accounted as complement inhibitory agents. Their derivatization with lysine greatly improved their solubility and made possible their *in vivo* application without the side effects proper for alcohol or dimethylsulfoxide solutions. The application of complexes in various *in vivo* models is a logical next step to evaluate their use in real immune processes and their possible use in practice.

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