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Antitussive activity of an extracellular *Rhodella grisea* proteoglycan on the mechanically induced cough reflex

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ABSTRACT

A mucilagineous extracellular proteoglycan (EPG) composed of xylose and its 3-*O*-and 4-*O*-methyl-derivates (55%), glucuronic acids (17%), rhamnose (14%), galactose (8%), glucose (4%) and minor amounts of other sugars (~2%) has been isolated from culture medium of *Rhodella grisea*. A white fluffy algal biopolymer of molecular mass over 8.1×10^5 contained protein (13%), methoxyl (6%), acetyl and succinyl groups. EPG was tested *in vivo* on mechanically induced cough in non-anaesthetized cats as a test system. The biopolymer showed a cough suppressing effect on laryngopharyngeal type of cough while the cough from tracheobronchial mucous area was slightly or not affected. Further, the intensity of maximal cough efforts from laryngopharyngeal and tracheobronchial parts in expirium and inspirium were influenced slightly only indicating that the expectoration effect was not suppressed by biopolymer application.

1. Introduction

The cough is an important, defensive pulmonary reflex which removes irritant fluids, or foreign materials from the airways. However, when cough is exceptionally intense or when it is non-productive and/or chronic it requires pharmacological suppression. For many patients old opioid drugs such as codeine or similar ones are still applied. These drugs suppress cough at equivalent doses, however, they produced significant ancillary liabilities such as respiratory depression (mainly in children), GIT disturbances (constipation), sedation, increasing viscosity and elasticity of mucus and this way worsening expectoration of phlegm from the airways (McLeod, Correll, Jia, & Anthes, 2008). Thus, the discovery of novel and effective antitussive drugs with an improved side effect profile relative to codeine would fulfil an unmet clinical need in the treatment of pathological cough reflex. In the last decades antitussive agents were enriched by natural compounds with different structure, size of molecules and mode of action. Relatively a wide class of natural compounds – alkaloids, flavonoids, saponins, tannins, terpenoids and carbohydrates are considered to induce antitussive and expectorans effects. Generally, antitussives represent a large scale of natural, semi-synthetic and synthetic compounds.

In the nineties Nosál'ová et al. (1992) discovered that mucilage, a viscous carbohydrate rich material isolated from a medicinal plant *Althaea officinalis*, showed an antitussive activity on mechanically induced cough reflex in non-anesthetized cats as a test system. More detailed analysis of *Althaea* mucilage revealed glucan, arabinan and rhamnogalacturonan polymers as its main structural components. Further antitussive activity tests of individual purified polysaccharide components of mucilage revealed the rhamnogalacturonan as the most active component to be responsible for antitussive effect of *Althaea* hydrocolloid (Nosál'ová, Strapková, Kardošová, & Capek, 1993). Later, acidic polysaccharides such glucuronoxylans, pectic polysaccharides and mostly rhamnogalacturonan type of polymers from *Salvia officinalis*, *Malva mauritiana, Verbascum thapsiforme*, *Rudbeckia fulgida* and *Mahonia aquifolium* were shown to be the most active carbohydrate components influencing the experimentally induced cough mainly from laryngopharyngeal airway area (Nosál'ová, Capek, Šutovská, Fraňová, & Matulová, 2006, chap. 52; Šutovská et al., 2009). It has been found that their high cough suppressive effect is connected mainly with decreasing of the cough effort numbers and the cough attacks intensity during the expiration and inspiration. Besides, the isolated herbal biopolymers did not influence the intensity of maximal cough efforts in the inspiration and expiration which are important parameters characterizing their expectoration quality. The high antitussive effect, no negative influence on expectoration and rare adverse reaction on organism make some herbal

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polysaccharides prospective drugs for their application in the treatment of cough.

The lowest plant organisms – algae, similarly as the higher plants, are rich source of low and high molecular mass compounds as are fatty acids, pigments, sterols, polysaccharides, glycoconjugates, etc. (Toncheva-Panova, Donchev, Dimitrov, & Ivanova, 2002). Their extracellularly produced polysaccharides or glycoconjugates due to their interesting rheological properties found use in some industrial applications as are food industry, pharmacology and medicine. Despite of the fact that a large scale of biological effects (antithrombic, antiviral, hypocholesterolemic, immunomodulatory and others) of extracellular biopolymers from different algae species was demonstrated there are no available data about their antitussive activity up to now (Capek, Matulová, & Combourieu, 2008). Therefore, the aim of the present paper was to verify the possible antitussive activity of a mucilagineous proteoglycan produced by unicellular fresh-water red alga *Rhodella grisea*.

2. Materials and methods

2.1. Material

The culture of the unicellular fresh-water red alga *R. grisea* was isolated from Piešťany spa thermal water, Slovakia. The strain HK 1983/1 is maintained at the culture collection CCAP at the Institute of Botany, Academy of Sciences of the Czech Republic, Třeboň.

2.2. Cultivation of R. grisea and isolation of extracellular glycoprotein

The inoculum of *R. grisea*, strain HK 1983/1 was cultivated in a liquid medium aerated with 1% CO_2 in air under continuous illumination and controlled temperature for two weeks (Fresnel, Billard, Hindák, & Pekárková, 1989; Pekárková, Hindák, & Smarda, 1988). At the stationary phase of growth the *R. grisea* cells were separated by centrifugation ($3000 \times g$ for 30 min) and extracellular biopolymers were recovered from culture medium by ethanol precipitation, dialysis and freeze-drying in 1988.

2.3. General methods

Solutions of carbohydrates were concentrated under diminished pressure at a bath temperature below 45 °C. Polysaccharides were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120 °C. The quantitative determination of the neutral sugars was carried out in the form of their trifluoroacetates by gas chromatography on a Hewlett-Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m), the temperature program of 110–125 (2 °C/min)–165 °C (20 °C/min) and flow rate of hydrogen 20 cm^3/min (Shapira, 1969). The uronic acid content was determined with the 3-hydroxybiphenyl reagent (Blumenkrantz & Asboe-Hansen, 1973). Elemental analysis was performed with EA 1108 apparatus (FISONS Instruments, United Kingdom). Protein was calculated from the nitrogen content (%N × 6.25). Gas chromatography–mass spectrometry of partially methylated alditol acetates as effected on a FINNIGAN MAT SSQ 710 spectrometer equipped with a SP 2330 column (0.25 mm × 30 m) at 80–240 °C (6 °C/min), 70 eV, 200 μA, and ion-source temperature of 150 °C (Jansson, Kenne, Liedgren, Lindberg, & Lönngren, 1976). HPLC measurement was performed with a Shimadzu apparatus (Japan) equipped with a differential refractometer RID-6A and a UV-vis detector SPD-10AV using a tandem of two columns HEMA-BIO 300 followed HEMA-BIO 1000 columns (8 mm × 250 mm) of particle size 10 μm. As a mobile phase 0.02 M phosphate buffer pH 7.2 containing 0.1 M NaCl was used at a flow rate 0.8 mL/min. A set of dextran standards was used for calibration of the column (Gearing Scientific, Polymer Lab. Ltd., UK).

2.4. Animals

Healthy awaken cats both sexes, weighing 1500–2500 g (8 in each group) were used. Cats were obtained from a domestic breeding. All animals were located in faculty animal house for one to two weeks of quarantine prior to experiments. Animals were kept in faculty animal house with food and water *ad libitum* and with a standard air conditioning system. The experimental protocol was complied with Slovakian and European Community regulations for use of laboratory animals in research and follows the criteria of experimental animal's well fare as well as local regulations and ethical considerations.

2.5. Method of the mechanically induced cough reflex

The experimental cough reflex was induced by mechanical stimulation of laryngopharyngeal and tracheobronchial parts of airway mucosa of the non-anesthetized cats of both sexes. After several days of quarantine, a tracheal cannula was surgically implanted into the trachea of experimental animals. This step of the experimental procedure was carried out under general anaesthesia, using thiopental (Thiopental inj., ICN Czech Republic) administered intraperitoneally in a dose 40 mg/kg body weight. Standard surgical care of operative wound was followed during next seven days. After this time the implanted chronical endotracheal canula served for the mechanical stimulation of the airways. A nylon fibre of 0.35 mm diameter as a mechanical stimulus was used and it was inserted into both the laryngopharyngeal and the tracheobronchial areas, subsequently moving it up and down (Nosál'ová, Strapková, Korpaš, & Crisciulo, 1989). The chronic tracheal cannula also provided of recording the side tracheal pressure.

The cough-related parameters were evaluated on the basis of intraluminal pressure changes recorded with a Mingograph (Elema device, Sweden) during the stimulation of the laryngopharyngeal (LP) and tracheobronchial (TB) mucosa part of the airways of vigilant cats. The number of cough efforts (NE, it indicates a response on the mechanical stimulation of the animal airways by nylon fibre), the cough frequency (NE min^{-1}), intensity of maximal cough efforts during expiration (IME^+) and inspiration (IME^-), intensity of cough attack during expiration (IA^+) and inspiration (IA^-) were evaluated. Prior to application of *Rhodella* biopolymer, the induced-cough parameters were recorded to get the control value (N) for each animal. The same parameters of experimental cough in time intervals of 0.5, 1, 2 and 5 h were registered after administration of *Rhodella* biopolymer in a dose 50 mg/kg body weight per orally. Codeine phosphate (Lachema, Brno, Czech Republic) was administered in a dose 10 mg/kg body weight orally.

2.6. Statistical analysis

Student's *t*-test and Wilcoxon–Wilcox test (Wilcoxon & Wilcox, 1964) were used for the statistical analysis of the obtained results. Data are presented as mean ± standard error of the mean (±S.E.M.). Asterisks mark statistical significant results ($p < 0.05^*$, $p < 0.01^{**}$ and $p < 0.001^{***}$). The $p < 0.05$ (*) and lower level of probability were considered as significant.

3. Results and discussion

Fresh-water red alga *R. grisea* was cultivated in a liquid medium aerated with carbon dioxide in air for two weeks (Fresnel et al., 1989; Pekárková et al., 1988). The microscopic observation during cultivation process showed light-green colonies of *Rhodella*

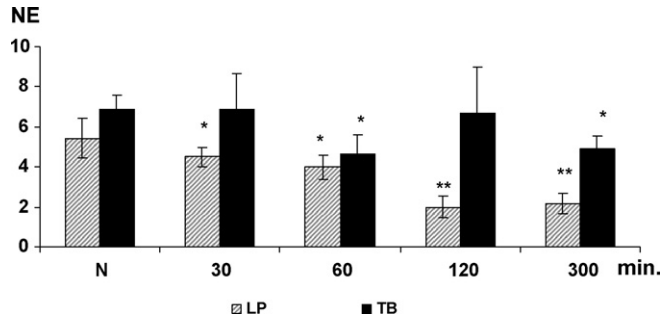

Fig. 1. The changes in the number of cough efforts (NE) after application of the mucilagineous extracellular proteoglycan (EPG) from *R. grisea* (50 mg/kg per os). Axis *x* – represents time intervals of the mechanical stimulation of the airways and axis *y* – represents the changes the number of the cough efforts. LP – laryngopharyngeal area, TB – tracheobronchial area, N – cough values recorded before EPG application. The columns present mean values of cough parameters, the range denotes standard error of means ± S.E.M. The significance 5% is marked by one asterisk, 1% by two asterisks.

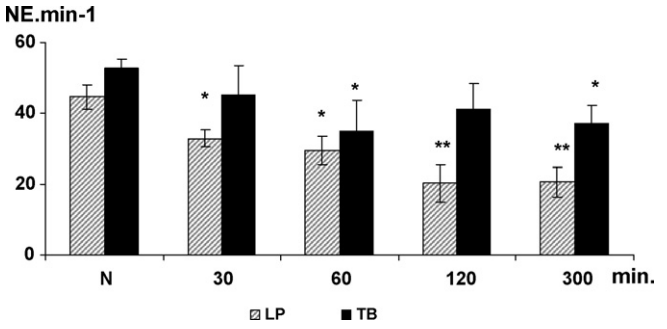

Fig. 2. The effect of extracellular proteoglycan (EPG) from *R. grisea* on the cough frequency (NE min^{-1}). Axis *x* – represents time intervals of the mechanical stimulation of the airways and axis *y* – represents the changes frequency of the cough. LP – laryngopharyngeal area, TB – tracheobronchial area, N – cough values recorded before EPG application. The columns present mean values of cough parameters, the range denotes standard error of means ± S.E.M. The significance 5% is marked by one asterisk, 1% by two asterisks.

cells surrounded by broad gelatinous polysaccharide matrix. The culture medium was centrifuged and the supernatant containing carbohydrates was concentrated and precipitated into 96% ethanol. Precipitate was removed by centrifugation, solubilized in water, dialyzed and freeze-dried to give a white fluffy extracellular material in the yield ~0.9 to 1.2 g/L of culture medium. Compositional analysis of extracellular material revealed the presence of carbohydrates and a moderate protein content (~13%), and indicated thus the proteoglycan nature of *Rhodella* extracellular material (EPG). Sugar analysis of EPG showed the presence of xylose and its 3-*O*- and 4-*O*-methyl-derivates (55%), glucuronic acids (17%), rhamnose (14%), galactose (8%), glucose (4%) and minority amounts of other sugars (~2%) i.e., mannose, fucose, and 2,3-di-*O*-methyldeoxyhexose. Further, biopolymer is substituted by methoxyl, acetyl and succinyl groups, and its average molecular mass was estimated to be 8.1×10^5. From the sugar analysis it is evident that the carbohydrate part of EPG is composed of partly methylated xylan based acidic heteropolysaccharide (Capek et al., 2008).

It has been found that mucilagineous rhamnogalacturonan isolated from *A. officinalis* roots was shown to be the most active antitussive agent of all carbohydrates tested (Nosál'ová et al., 2006). The high antitussive activity of *Althaea* hydrocolloid was ascribed due to its viscous properties. Similarly, *Rhodella* EPG forms in water extremely viscous hydrocolloid. This fact motivated us to verify a possible antitussive effect of algal proteoglycan. Antitussive activity tests were performed *in vivo* conditions in the test systems – non-anaesthetized cats. The cough-suppressing effect of EPG was investigated after mechanical stimulation of the laryngopharyngeal (LP) and tracheobronchial (TB) area of the airways. Viscous water solution of EPG (2 mL) was applied orally in a dose 50 mg/kg body weight of animals tested and cough parameters were recorded using a Mingograph.

The administration of EPG resulted in a statistically significant reduction number of cough efforts (NE) predominantly from LP mucosa area of the airways (Fig. 1). The first significant reduction of cough efforts was recorded within 30 min indicated on prompt onset of the action, however, the most significance decrease of this parameter was noticed after 2–5 h after EPG application. As can be seen in Fig. 1, the influence of EPG on the number of cough efforts from TB mucosa area was less marked. Similarly, EPG significantly influenced the cough frequency (NE min^{-1}) mainly from LP area (Fig. 2). The value of the cough frequency decreased within 30 min after administration of EPG and the highest effect was noticed after 2 h and lasted during the entire experiment course. From Fig. 2 it is evident that TB area of the airways was less affected, similarly as in the case of cough efforts.

The next cough parameters, i.e. intensity of maximal cough efforts during expiration (IME^+) and inspiration (IME^-) from LP and TB areas of the airways were not influenced significantly by EPG application (therefore data are not shown). This fact is important from pharmacological point of view.

The significant suppression of the intensity of the cough attacks in expirium (IA^+) from LP mucosa area of the airways after EPG administration has been noticed (Fig. 3). This parameter of cough reflex was significantly decreased from LP mucosa area after 30 min and suppression effect lasted during the entire experiment course.

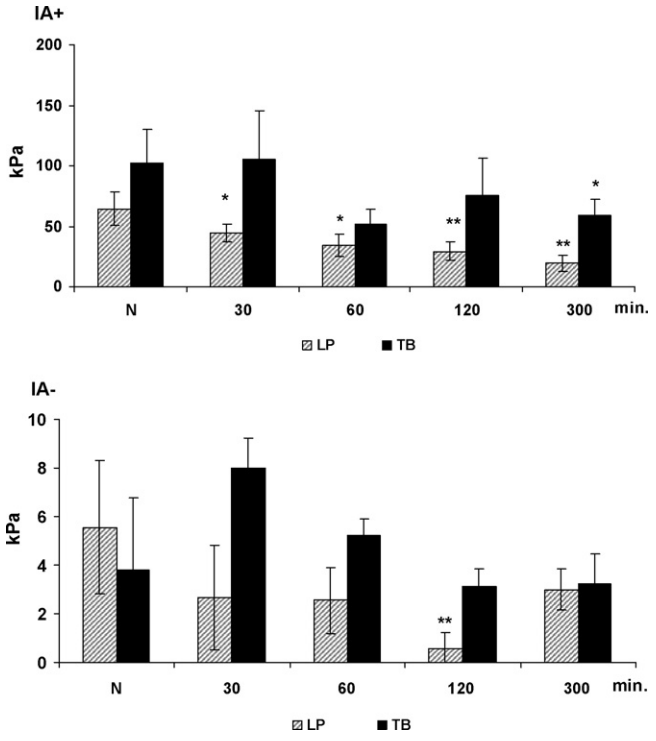

Fig. 3. The effect of extracellular proteoglycan (EPG) from *R. grisea* on intensity of cough attacks during expiration (IA^+) and inspiration (IA^-). Axis *x* – represents time intervals of the mechanical stimulation of the airways and axis *y* – represents the changes intensity of cough (IA^+ – expiration, IA^- – inspiration) expressed as a kilopascal (kPa). LP – laryngopharyngeal area, TB – tracheobronchial area, N – cough values recorded before EPG application. The columns present mean values of cough parameters, the range denotes standard error of means ± S.E.M. The significance 5% is marked by one asterisk, 1% by two asterisks.

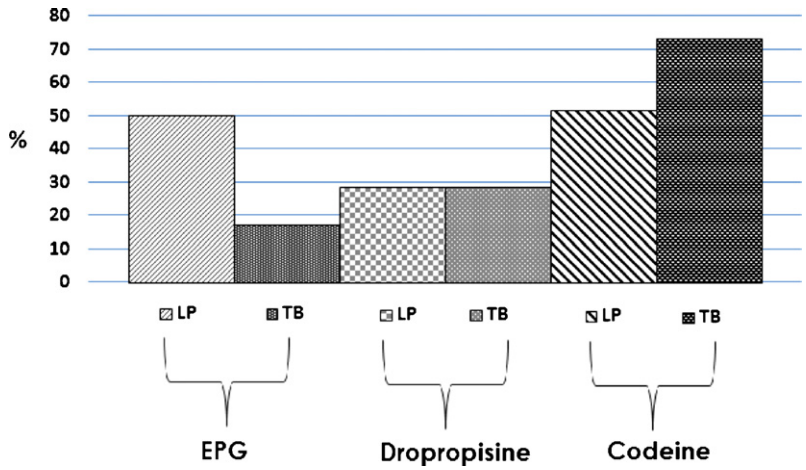

Fig. 4. The percentage statements of the cough suppressive activity of the extracellular proteoglycan (EPG) from *R. grisea* (50 mg/kg b.w.), dropropisine (100 mg/kg b.w.) and codeine phosphate (10 mg/kg b.w.). LP – antitussive efficiency on the cough from laryngopharyngeal area, TB – antitussive efficiency on the cough from tracheobronchial area of the airways.

However, TB area was significantly influenced only after 5 h from EPG application. The intensity of the cough attacks in inspirium (IA^-) from LP areas was significantly inhibited only 2 h after EPG application while TB area was slightly or not statistically affected (Fig. 3). A comparison of the cough suppressive activity of the mucilagineous proteoglycan (EPG) with classical and in clinical practice used drugs both nonnarcotic (dropropisine) and narcotic antitussive group (codeine) is shown in Fig. 4.

From the experimental data it is evident that peroral administration of EPG suppressed cough reflex in conscious cats tested. It brought about a significantly decrease in number of cough efforts (NE), frequency of cough (NE min^{-1}) and the intensity of the cough attacks in expirium (IA^+) and inspirium (IA^-) mainly from LP mucosa area of the airways. Moreover, all followed cough parameters (except the intensity of the cough attacks in inspirium) were suppressed significantly within 0.5 h after administration of the tested compound and this decrease lasted during the entire experiment course. It has been found that EPG had slight or no effect on TB mucous area. These results are surprising and have important pharmacological implication. Generally it is known that (i) the cough induced by stimulation of LP mucous area of the airways (in comparison with the cough from TB mucous area) is very difficult to suppress by common antitussive agents and (ii) mechanically induced cough reflex (unlike those with chemical irritants) is resistant to antitussive agents (Korpáš & Tomori, 1979). A few drugs are known only that are able to suppress the laryngopharyngeal type of pathological cough reflex till now (Korpaš, Paintal, & Anand, 2007). Antitussive drugs are more active against the tracheobronchial type of cough. The experimental results showed that cough suppressive activity of EPG on the cough from LP mucous area exceeded antitussive potency of dropropisine, a representative of nonnarcotic antitussive agents commonly used in a clinical practice. Further, its antitussive activity on the cough from LP area is comparable with that of codeine, the most effective suppressant of cough reflex. It seems that the ability of *Rhodella* EPG to suppress cough from LP mucous area of the airways could be connected with its mucilagineous character. After administration of EPG into the oral cavity it can form a protective film on mucosa areas upper parts of the airways and thus may decrease the sensitivity of cough receptors to irritation. Covering mucosa airways with the viscous film evokes a significant protection of peripheral nervous "sensor" for cough stimuli mainly in the larynx. This assumption can be supported by the fact that *Rhodella* proteoglycan forms gelatinous solutions due to its structure and variety of functional groups (carboxylic, methoxyl, acetyl and succinyl) located along its carbohydrate backbone. Moreover, EPG possess a high water-binding capacity and due to a relative high carboxyl content it can participates in cation exchange processes. Mutual interactions of EPG with mucous can finally resulted in a decreasing irritation of airways and suppression of pathological cough reflex.

4. Conclusion

It can be concluded that proteoglycan complex (EPG) isolated from cultural medium of red alga *R. grisea* has been found as a significant antitussive agent influencing the cough from laryngopharyngeal mucous area of the airways while the cough from tracheobronchial mucous area was negligibly affected. This finding is important due to the fact that it is very difficult to suppress laryngopharyngeal type of cough by common antitussive agents. Most of antitussive acting drugs used in clinical practice influence or suppress predominantly the cough from tracheobronchial mucosa area of the airways and many of them (e.g. codeine) have a negative effect on expectoration process. It has been shown that *Rhodella* proteoglycan was able to influence all parameters characteristic for cough symptoms, i.e. number of cough efforts, frequency of cough and the intensity of the cough attacks in expirium and inspirium from laryngopharyngeal mucosa area. However, the intensity of maximal cough efforts during expiration and inspiration from laryngopharyngeal and tracheobronchial areas of the airways were influenced slightly only. This fact is important from the pharmacological point of view and indicates that the expectoration effect was not suppressed by proteoglycan tested. Due to the specific antitussive effect, *Rhodella* proteoglycan could be considered as a prospective drug for possible human antitussive therapy, however, an additional pharmacological research in this field is required. It seems that the specific effect of this biopolymer results from the primary structure of its polymeric chains, i.e. the type, the size and the sequence of monosaccharide components, the anomeric configuration of glycosidic linkages and the presence of the functional groups which are parts of the molecule. All these structural elements contribute to its excellent mucilagineous properties which predetermine this biopolymer for perspective industrial applications.

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