

THE FLOW CYTOMETRIC ANALYSIS OF LUPIN PROTEINS' POTENTIAL TO INDUCE THE RESPIRATORY BURST IN HUMAN NEUTROPHILS

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Abstract. There is an ever-increasing demand for lupin seed protein in human nutrition. However, their usage as a food ingredient can be limited by their influence on the immune system. The aim of the study was to analyse, by means of flow cytometry, the potential of lupin seed globulins to induce the respiratory burst in human neutrophils. The results proved the potential of lupin globulins to induce the increased ROS production in human neutrophils. The increased ROS production by the cells treated with lupin globulins and, at the same time, stimulated with the PMA may suggest a synergistic effect of the globulins and the PMA.

Key words: lupin globulins, immunoreactivity, respiratory burst, reactive oxygen species, neutrophils, phorbol 12-myristate 13-acetate

INTRODUCTION

Lupin is increasingly being used as a valuable ingredient in various food products, functional food products among them. The lupin protein provides both excellent nutritional value, comparable to this of soybean, and useful technological functionality to the foods to which it is added [Arnoldi 2005, Bez et al. 2005, Papoti et al. 2005, Lampart-Szczapa et al. 2006]. The investigation carried out on an animal model as well as pilot clinical studies have proved the lupin-containing foods' beneficial effect on circulatory system [Naruszewicz 2005].

However, there have been a number of reports worldwide of the possible adverse impact of lupin protein on the human immunological system. Most of the reports concern the ability of lupin protein to cause food allergy or food intolerance in people after consuming lupin-fortified products [Faeste et al. 2004, Magni et al. 2005, Radcliffe et al. 2005, Rojas-Hijazo et al. 2006, Dooper et al. 2007].

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So far there has not been any information about any other kinds of lupin protein effect upon immunological mechanisms. Nevertheless, it is a well-known fact that some plant proteins affect the immunological system response by the activation of neutrophil granulocytes [Brando-Lima et al. 2005, Stein et al. 2005]. Neutrophils are highly specialized white blood cells, which take part in the effector phase of immunological response. During this phase reactive oxygen species (ROS) and oxidised halogens are generated in a process which is called respiratory burst [Wientjes and Segal 1995, Hampton et al. 1998, Dahlgren and Karlsson 1999, Burg and Pillinger 2001].

The aim of the study was to analyse the ability of lupin globulins to stimulate ROS production in human neutrophils, in order to complete the characteristics of lupin protein immunoreactivity. To achieve this, a method of the cytometric analysis of neutrophils' respiratory burst was applied.

EXPERIMENTAL PROCEDURES

Lupin globulins were isolated [Freitas et al. 2000] from lupin flour (*Lupinus angustifolius*, var. Baron), supplied by the Institute of Food Technology of Plant Origin (Poznań University of Life Sciences), and fractionated by means of gel filtration chromatography (glass column packed with Sephadex G 200, Labart). Three globulin fractions with the protein concentration [Bradford 1976] of 676 µg/ml, 177 µg/ml and 144 µg/ml, respectively, were numbered from one to three and subjected to some further analysis. Peripheral blood samples (2.5 ml each) of twelve healthy donors (Provincial Blood Donation Station, Poznań), were collected onto lithium heparin granules (Primavette®), (KABO Labortechnik). The research protocol was approved by the Bioethical Commission of University of Medical Sciences in Poznań.

The intensity of respiratory burst was measured with the use of dihydrorhodamine 123 (123 DHR), [Sigma, USA], which was oxidized intracellularly to rhodamine 123 by hydrogen peroxide [Vowells et al. 1995]. Rhodamine, a fluorescent dye, can be detected and measured by a flow cytometer.

As oxygen metabolism in resting cells is on relatively low level, we used phorbol-12-myristate-13-acetate (PMA), [Sigma, USA], which is a potent stimulant of respiratory burst and ROS production.

Globulin fractions one to three, suspended in 50 mM Tris-HCl buffer (pH 7.5), were added (10 µl per sample) to test tubes containing 50 µl of heparinised blood. In the control samples, the blood was treated only with Tris-HCl buffer, without the addition of the analysed globulin fractions, or was left without the addition of the buffer or the globulins. All the samples were mixed thoroughly and incubated for 30 min, in room temperature, in the dark.

After this time, 25 µl of dihydrorhodamine 123 solution was added to each sample. The samples were then mixed and incubated for 5 min, in 37°C, in a cell culture incubator [Kebo Assab AB, Sweden], in the dark. After that, half of the samples were treated with 2 µl of the PMA (PMA-stimulated samples) and incubated for 15 min, in room temperature, without light. The remaining samples were left PMA-unstimulated. After the incubation period, 1000 µl of lysing solution [Becton Dickinson, USA] were added to all the samples, in order to stop the reaction and to remove the contaminating eryth-

rocytes. The process of erythrocyte lysis was carried out for 10 min, in room temperature, in the dark.

The measurement of rhodamine 123 fluorescence was performed by means of FAC-Scan flow cytometer [Becton Dickinson, USA]. ROS production was measured in each sample in 5000 neutrophils.

Basing on the light scatter (forward scatter and side scatter values) the cells were separated with respect to their size, shape and granularity on the 2D-graph with linear scale. On that base, the populations of peripheral blood leukocytes (lymphocytes, monocytes and granulocytes) were identified and the population of neutrophils was distinguished.

The measurement of rhodamine 123 fluorescence was performed at green spectrum of light (515-545 nm), using logarithmic signal amplification [Vowells et al. 1995]. Data was presented on histograms and expressed as the mean \pm S.D., calculated by the CellQuest software [Becton Dickinson, USA]. To evaluate the respiratory burst we compared the mean value of fluorescence of stimulated and resting neutrophils.

All data was analysed using Statistica 6.0 [StatSoft, USA]. The Wilcoxon matched pairs test was utilized to compare investigated and control samples. A p value less than 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

The analysed samples were numbered from one to ten and described as follows:

- 1 – control samples, PMA-unstimulated
- 2 – control samples, PMA-stimulated
- 3 – control samples with the addition of Tris-HCl buffer, PMA-unstimulated
- 4 – control samples with the addition of Tris-HCl buffer, PMA-stimulated
- 5 – investigated samples, globulin fraction one, PMA-unstimulated
- 6 – investigated samples, globulin fraction one, PMA-stimulated
- 7 – investigated samples, globulin fraction two, PMA-unstimulated
- 8 – investigated samples, globulin fraction two, PMA-stimulated
- 9 – investigated samples, globulin fraction three, PMA-unstimulated
- 10 – investigated samples, globulin fraction three, PMA-stimulated.

The results of the study showed an increased activity of neutrophils after treating them with lupin globulins. This phenomenon was indicated by the elevated ROS production in all the investigated samples (Table 1). Such a regularity in ROS generation was observed in the cells stimulated with the PMA (pairs of variables: 2&6, 2&8, 2&10) as well as in the PMA-unstimulated neutrophils, treated only with lupin globulin fractions (pairs of variables: 1&5, 1&7, 1&9).

Incubation of the samples only with Tris-HCl buffer did not cause any statistically significant changes in reactive oxygen species production, whereas incubation of the cells with the investigated proteins caused statistically significant changes in ROS production (Fig. 1 and 2).

The obtained results proved the potential of the investigated globulin fractions to induce the increased ROS production in human neutrophils. They also suggested the positive correlation between lupin globulin concentration and the elevated ROS generation

Table 1. Lupin globulin influence on ROS generation in neutrophils (the Wilcoxon matched pairs test)

Pairs of variables	Significant level $p < 0.05000$
1 & 3	0.307
1 & 5	0.005
1 & 7	0.012
1 & 9	0.006
2 & 4	0.388
2 & 6	0.002
2 & 8	0.008
2 & 10	0.012

by neutrophils. Such an elevated generation of reactive oxygen species in the cells treated only with lupin globulins, without the addition of the PMA, indicates that the analysed proteins are potent stimulators of respiratory burst. The increased ROS production by the cells treated with lupin globulins and, at the same time, stimulated with the PMA may suggest a synergistic effect of the globulins and the PMA.

Respiratory burst is one of the intracellular mechanisms of killing microbes. Production of reactive oxygen species by neutrophils, as a part of immune defense against pathogens, is an advantageous process. However, if ROS escape from a phagosome, they are potentially toxic to the surrounding cells [Wientjes and Segal 1995, Burg and Pillinger 2001, Król and Konopka 2003].

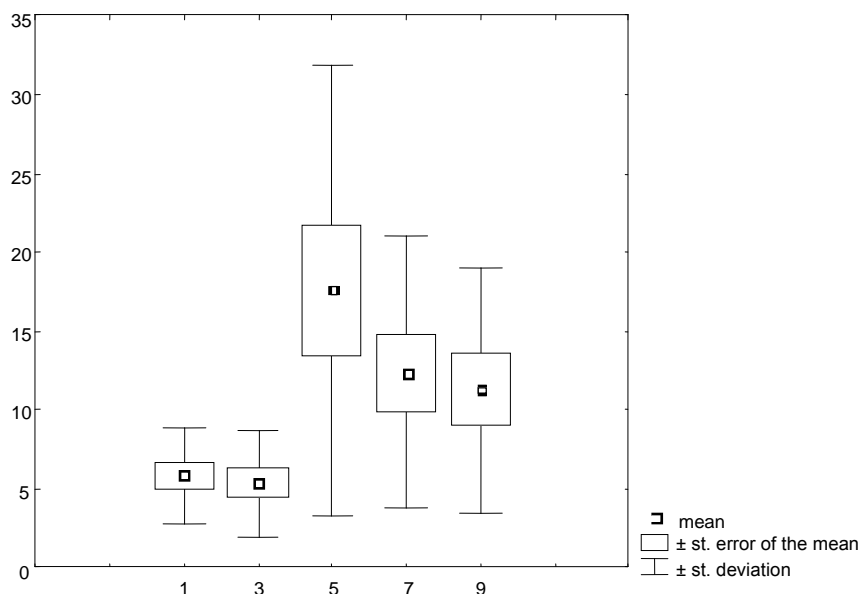


Fig. 1. The comparison of ROS production in PMA-unstimulated samples

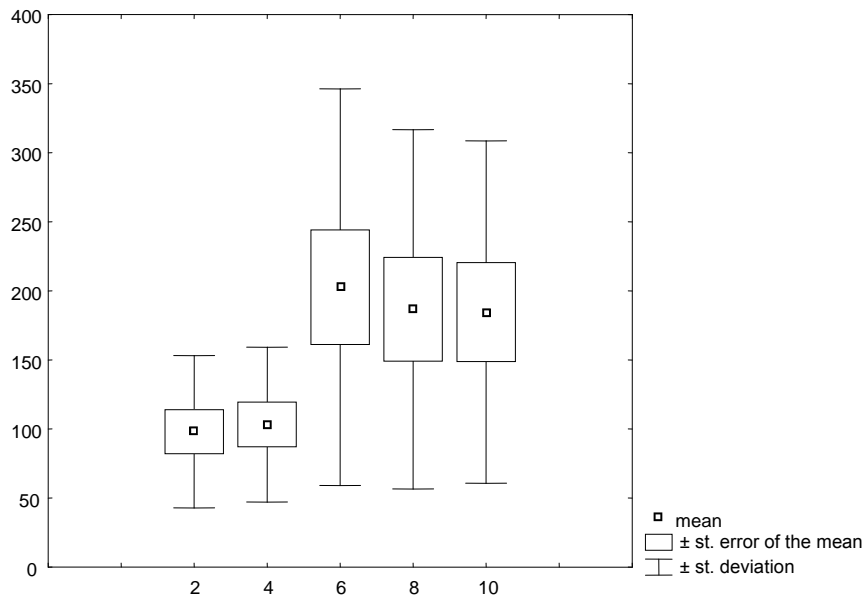


Fig. 2. The comparison of ROS production in PMA-stimulated samples

The increased ROS generation may also be the effect of some substances, like lectins or peptides, that bind sugar residues present on the surface of immunological cells. Such substances can bind unspecifically to the neutrophil surface receptors and initiate their respiratory burst [Brando-Lima et al. 2005, Stein et al. 2005]. This is an adverse phenomenon, especially when caused by compounds that are commonly regarded as innocuous or neutral to the organism. Similarly, lupin protein, which is desirable for its high nutritional value and widely used as a food ingredient, can at the same time have a harmful impact on human immunological system. The allergenic potential of lupin protein has been partly recognised and documented in the number of reports worldwide [Faeste et al. 2004, Magni et al. 2005, Radcliffe et al. 2005, Rojas-Hijazo et al. 2006, Dooper et al. 2007]. The cytometric analysis of ROS generated by lupin globulin-treated neutrophils, performed for the first time, proves that not only can lupin protein be an allergenic factor but also it can have an adverse impact on human neutrophils.

CONCLUSION

The three investigated lupin globulin fractions are potent stimulators of respiratory burst in human neutrophils. The elevated ROS production is observed in the cells stimulated with lupin globulins, as well as in the granulocytes stimulated with the globulins, along with PMA.

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**CYTOMETRYCZNA ANALIZA POTENCJAŁU BIAŁEK ŁUBINU
DO STYMULOWANIA WYBUCHU ODDECHOWEGO
W NEUTROFILACH CZŁOWIEKA**

Streszczenie. Białka z nasion łubinu znajdują coraz większe zastosowanie w żywieniu człowieka. Ich wykorzystanie jako składnika żywności może być jednak ograniczone wpływem tych białek na układ immunologiczny. Celem badania było przeanalizowanie, za pomocą cytometrii przepływowej, potencjału globulin z nasion łubinu do indukowania wybuchu oddechowego w neutrofilach człowieka. Uzyskane wyniki dowiodły zdolności globulin łubinu do stymulowania zwiększonej produkcji reaktywnych form tlenu przez ludzkie neutrofile. Zwiększone generowanie ROS przez komórki poddane działaniu globulin łubinu i jednocześnie stymulowane PMA może sugerować synergistyczne działanie globulin w stosunku do PMA.

Słowa kluczowe: globuliny łubinu, immunoreaktywność, wybuch oddechowy, reaktywne formy tlenu, neutrofile, 12-mirystynian, 13-octan forbolu

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