THE INHIBITION OF HUMAN PLATELET AGGREGATION BY A LOW-MOLECULAR WEIGHT CHITOSAN

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Abstract

Chitosan derivatives were obtained by chemical (MW of 6 kDa, DD 99% - Ch6/99; MW13 kDa, DD 98% - Ch13/98) and enzymatic (MW of 5 kDa, DD 85% - Ch5/85; MW of 10 kDa, DD 85% - Ch10/85) depolymerisation of chitosan with a MW of 334 and 1000 kDa. Chitosan derivatives (almost identical MW pairs and different DD) possessed insignificant anticoagulant activity, did not promote human platelet aggregation and reduced ADP or collagen-induced platelet aggregation. The studied samples at a concentration of 2 mg/ml reduced the aggregation of platelets more than twice induced in $2x10^{-6}$M and $1x10^{-5}$M concentrations; at weak activation in $2x10^{-6}$M, the Ch10/85 sample was the most effective. The Ch6/99 and Ch13/98 samples were 20 times more effective at the inhibition of collagen-induced platelet aggregation than the Ch10/85 sample. The latter can be explained by the greater value of positive charge (DD) and polydispersity (M_w/M_n) of chitosan samples obtained by chemical depolymerisation.

Keywords: *chitosan, depolymerisation, molecular weight, deacetylation degree, platelet aggregation*

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1. Introduction

Chitosan is a natural polymer with low toxicity, a compatibility of degradation products with living organisms, and may be a prerequisite for its use in preclinical and clinical trials [1]. However, nowadays, there is no authorisation to use chitosan in the medical field (USFDA), but the derivatives ─ chitosan hydrochlorides ─ were previously included in the European Pharmacopoeia in 2002 [2]. One of the major reasons which limits the study of chitosan in the biomedical field is the lack of solubility at pH values close to neutral. At pH<3, the amino groups in the polymer structure are almost completely protonated, and the chitosan acquires the characteristics of a water-soluble cationic polyelectrolyte. The solubility of chitosan depends on the degree of deacetylation (DD), the distribution of acetyl groups along the polymer chain and the molecular weight (MW). The reduction in molecular weight improves the polymer solubility at pH 5.5–6.5. Low molecular weight chitosan (LMWCh) and its oligomers can be obtained by chemical, physical or enzymatic depolymerisation of the polymer [3]. Chemical depolymerisation is usually carried out by adding an inorganic acid ─ HCI [4] ─ or using either HNO_2 or H_2O_2 [5]. The physical method incorporates different types of radiation [6,7]. In the enzymatic method, the application of commercial enzymatic products is preferred [8]. Clearly, the method responsible for the depolymerisation of chitosan affects the main physicochemical properties (MW, DD) as well as the reaction products formed and the biological activity performed [9,10].

A number of authors showed the influence of molecular weight and the degree of substitution of chitosan samples with platelets and plasma haemostasis (haemostasis is a system of mammalian organisms providing a liquid blood state, stopping bleeding at locations of damage to the vascular wall and fibrinolysis – the dissolution of blood clots) [11,12]. The basis of the mechanism of action of drugs from groups of anti-platelets and anticoagulants (used for the prevention and treatment of arterial or venous thrombosis, leading to strokes, heart attacks, etc. [13,14]) is the inhibition of platelet aggregation and the inhibition of coagulation factors, or antithrombin activation, a plasma inhibitor of coagulation factors. In recent years, many publications devoted to an increase in thromboresistance (a decrease in the risk of blood clot development) on the chitosan modified surface of the polymeric materials making contact with blood have been released [15,16].

The purpose of this study is to analyse the influence of the low-molecular weight chitosan obtained by chemical and enzymatic depolymerisations on platelet aggregation and human plasma/blood coagulation times.

2. Materials and Methods

High molecular weight chitosan (HMWCh) with a MW of 1000 kDa and DD of 85% was used ("Bioprogess", Russia) in this work. The depolymerisation of HMWCh was carried out using a dry enzyme preparation obtained by lyophilic drying of the culturing liquid of the *Myceliophthora fergusii* mycelial fungus strain VKM F_3932D (All-Russian Collection of Microorganisms (VKM), G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS). The enzymatic hydrolysis of chitosan was carried out as outlined in the procedure [17]: 1.0 g of further purified chitosan (DD 85%) was dissolved in 40 ml 1.0 M acetic acid solution with stirring, then 116 ml of water was added. The resulting solution was basified with 1.0 M NaOH (44 mL) to pH 5.4–5.6, held at 37°C, and a dry enzyme preparation (20 mg, enzyme/substrate ratio of 1/800) was added. Enzymatic hydrolysis was carried out for 0.5-2 hrs with stirring. The reaction was stopped by boiling for 10 min. The products of hydrolysis from the reaction medium were isolated by re-precipitation with a 12% solution of NH_4OH. The resulting suspen-

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sion was centrifuged (5000g, 30 min), and the pellet was re-suspended in water, dialysed and lyophilised. The yield of chitosan samples with a free amino group as a function of MW was 40–50%. LMWCh samples were obtained as a result of chemical depolymerisation from chitosan with a MW of 334 kDa, and DD of 96%, as outlined in the procedure [18].

The blood from an elbow vein of donors stabilised by 0.11 M sodium citrate solution (the blood was selected to a tag in a plastic syringe of S-Monovette 5ml 9NC "Sarstedt", Germany) was used in this work, as well as human plasma, both rich in and depleted of platelets (National Research Centre for Haematology of the Ministry of Healthcare of the Russian Federation; all donors gave the written informed consent for the collection and use of their blood). The blood was centrifuged for 7 min at 150 g and room temperature in order to obtain platelet-rich human plasma. Stabilised human blood was centrifuged for 20 min at 1300 g to obtain-platelet-deficient plasma. Then, 0.05 M Tris-HCl buffer with 0.175 M of NaCl, pH 7.4, was used to dissolve substances.

The effect of the studied compounds on the coagulation time of stabilised human blood was evaluated using the blood re-calcification time test (BRT) [19]: 0.1 ml of 0.02 M $CaCl_2$ (Sigma) was added to 0.1 ml of the blood containing chitosan derivatives at a concentration of 20 mg/ml and the time of appearance of the clot was recorded using a coagulometer. Blood coagulation time in activated partial thromboplastin was determined by analysing the effects of compounds on the coagulation of platelet-deficient human plasma [20] (APTT: 0.1 ml mixture of ellagic acid and phospholipids was added to 0.1 ml of the human plasma containing chitosan derivatives at a concentration of 0.036–1.790 mg/ml; 0.1 ml solution of 0.025 M $CaCl_2$ was added after 3 min of incubation at 37ºC and the time to the appearance of clots was recorded using a coagulometer) as well as prothrombin time [21] (PT: 0.1 ml of plasma containing chitosan derivatives in 0.36 and 3.58 mg/ml concentrations was incubated for 1 min at 37°C; then 0.2 ml of Ca^{+2}-thromboplastin solution was added and the time of appearance of the clot on a coagulometer was recorded by NPO “Renam” kits (Moscow, Russia). When calculating the antithrombin activity (aIIa), the APTT test system for unfractionated heparin (UFH, aIIa = 160 IU/mg, republican unitary manufacturing enterprise “Belmedpreparaty”, the Republic of Belarus, Minsk) was calibrated and compared to the indications when adding compounds to the plasma. The time of appearance of the clot (in 4–10 independent determinations) on a 2-channel opto-mechanical coagulometer APG2-01 Minilab 701 (JSC SPE "Technomedica", Moscow, Russia) was recorded.

The influence of chitosan derivatives on platelet aggregation was determined by Born's method [22], using a Model 500 aggregometer ("Chrono-Log", USA) with the recorder capturing changes in the light transmission of platelet-rich human plasma. A solution of disodium adenosine-5'-diphosphate salt (ADP; "Sigma-Aldrich") or collagen (scientific and production association “Renam”, Moscow, Russia) was used as an inductor of aggregation. Platelet-rich human plasma (0.450 ml) was incubated (1 min at 37°C) with the studied derivatives (from 0.26–1.37 mg/ml), then ADP ($2x10^{-6}$–$2x10^{-5}$ M) or collagen (50 μ/ml) was added. Plasma with the addition of 0.045 ml of 0.05 M tris-HCl buffer, pH 7.4, containing 0.175 M NaCl was used as a control. Platelet aggregation was recorded for 5 min (the light transmission of the human platelet-deficient plasma was taken to be 100%). The maximum amplitude of the aggregation curve was determined on the aggregogram (platelet aggregation curve) as a percentage.

The results are presented by arithmetic mean values with standard average arithmetic errors from 5 to 10 independent determinations. The reliability of the differences between indications ($p < 0.05$) was estimated by Kruskal-Wallis criterion. Statistical processing of the results was carried out using the Primer of Biostatistics Version 4.03 program.

3. Results and Discussion

3.1. Preparation of chitosan samples

MW and DD have an effect on the solubility of chitosan at pH 7.2–7.4 in an aqueous solution. As the MW of chitosan decreases and DD increases, its solubility at pH values close to neutral usually increases. When depolymerising HMWCh, depending on the method applied, it is possible to obtain chitosan samples with similar characteristics ─ MW and DD, but different polydispersity or location of acetyl groups in chitosan oligomers. To study the effect of LMWCh on the aggregation of human platelets, samples of chitosan with a MW of 5 or 10 kDa were obtained by two different methods. Commercially available hydrolases with non-specific activity against chitosan are currently used to produce LMWCh. Hydrolases are able to show chitin- and chitosanolytic activity regardless of the mechanism of catalytic action [23]. A dry enzyme preparation with chitinase and chitosanase activity was used in this work to obtain LMWCh of 10 and 5 kDa. The benefits of choosing this enzyme preparation are the conservation of the natural structure of chitosan, the absence of deacetylation during the depolymerisation of the polymeric chain and the relatively low polydispersity index of the samples obtained. In addition, during the biological studies, LMWCh was used, which was prepared by chemical depolymerisation. The samples of LMWCh, prepared by both methods, were shown to have similar values of MW, but different DD and Mw/Mn. The physicochemical properties of the obtained samples of chitosan are listed in Table 1.

Table 1. Characteristics of the chitosan samples.

Samples	MW, kDa	M_w/M_n	DD, %
Ch6/99	6	2.54	99
Ch13/98	13	2.48	98
Ch5/85	5	2.00	85
Ch10/85	10	2.00	85

Note: the original chitosan "Bioprogress" from crab shells (MW 334 kDa, DD 96%) was hydrolysed with 0.6 M HCl [18], and samples of Ch6/99 and Ch13/98 were obtained; the initial chitosan "Bioprogress" from crab shells (MW 1000 kDa, DD 85%) was hydrolysed by the enzyme preparation from *Myceliophthora fergusii* [17] - samples Ch5/85 and Ch10/85.

3.2. Influence of chitosan samples by the human plasma/blood coagulation in the BRT, APTT and PT tests.

Some drugs, used for the prevention and treatment of thromboses in humans, reduce the mammalian plasma or blood coagulation time in standard coagulation tests [13,14]. Therefore, in the present work, it was decided to estimate the influence of chitosan on plasma coagulation using two tests. Fig. 1a and 1b demonstrate a significant increase in coagulation time in human plasma compared to the control in APTT and PT tests with the addition of oligochitosans Ch6/99 and Ch13/98 (obtained by acid hydrolysis) and Ch5/85 and Ch10/85 (obtained by enzymatic hydrolysis). The antithrombin activity of the samples was insignificant (Ch6/99, Ch13/98, Ch5/85 and Ch10/85 – 0.18±0.02, 0.25±0.03, 0.09±0.01 and 0.10±0.01 IU/mg, respectively). The addition of the studied oligochitosans (20 mg/ml) to the human blood stabilised by sodium citrate (BRT after

incubation for 10 min at 37ºC) led to the significant increase in time to coagulation in the BRT test (for Ch6/99, Ch13/98, Ch5/85 and Ch10/85 by 3.6, 2.8, 1.7 and 2 times, respectively) (Fig. 1c). It was shown that Ch6/99 and Ch13/98 possessed significantly lower anticoagulant activity in comparison with the samples of Ch5/85 and Ch10/85. However, the values of the antithrombin activity of the studied samples are extremely insignificant (the antithrombin activity of UFH is 150–250 IU/mg).

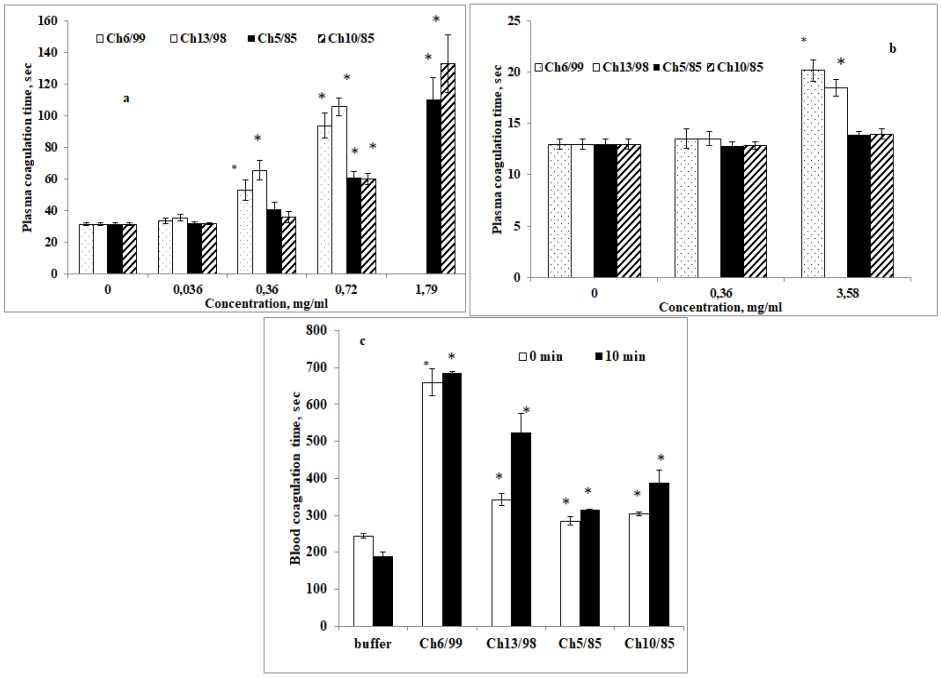

Figure 1. Influence of chitosan derivatives on the human plasma coagulation time in APTT (a) and PT (b) tests, and on the stabilised blood coagulation time in the BRT test (c). Concentration of substances in (c) 20 mg/ml; * - (P <0.05) the reliability of the differences with the indications in controls: for (a) and (b) – 0 mg/ml, for (c) – the buffer; n = 4–10.

3.3. Influence of chitosan samples on human platelet aggregation induced with ADP or collagen

A number of authors have shown that, depending on the structural parameters and the source of raw materials, chitosan can possess both pro-aggregation [24,25] and anti-aggregation activity. ADP (stimulates purine platelet receptors of P2Y; aggregation of platelets occurs without secretion of granules), and collagen (the activation by collagen triggers the aggregation, synthesis and secretion of thromboxane A2 from granules of platelets; collagen interacts with GPVI and GPIa/IIa receptors) were chosen as inductors to analyse the influence of chitosan derivatives on platelet aggregation in this work [26].

In the present work, the addition of samples of Ch6/99, Ch13/98, Ch5/85 and Ch10/85 to the human platelet-rich plasma did not lead to the induction of aggregation (Fig. 2c); significant differences with indications during incubation with the buffer (2.75±0.37%) were not observed (0.42–3.25%). After the addition of $2x10^{-5}$ M ADP during the induction of aggregation, a decrease in aggregation degree of platelets with an increase in the concentration of chitosan was noted; however, significant differences

from the control were only seen for the addition of samples at concentrations of 1 and 2 mg/ml (Fig. 2b and 2c). Ch6/99 and Ch13/98 reduced the aggregation of platelets by only 40–45% (in relation to 100% control). Polyakova et al. suggest that chitosan (structural parameters were not specified) significantly reduced the degree of ADP-induced platelet aggregation by only 11–18% [27]. In addition, disaggregation to 14% was observed after the peak of platelet aggregation was reached (with the addition of 2 mg/ml of Ch6/99 or Ch13/98). In order to reduce the aggregation of Ch5/85 and Ch10/85 samples by two times, 0.85 and 0.83 mg/ml was required (fig. 2c; disaggregation degree averaged 42 and 26%, respectively). Therefore, with a strong activation of platelet aggregation by ADP, the equal inhibitory efficiency of Ch5/85 and Ch10/85 was observed.

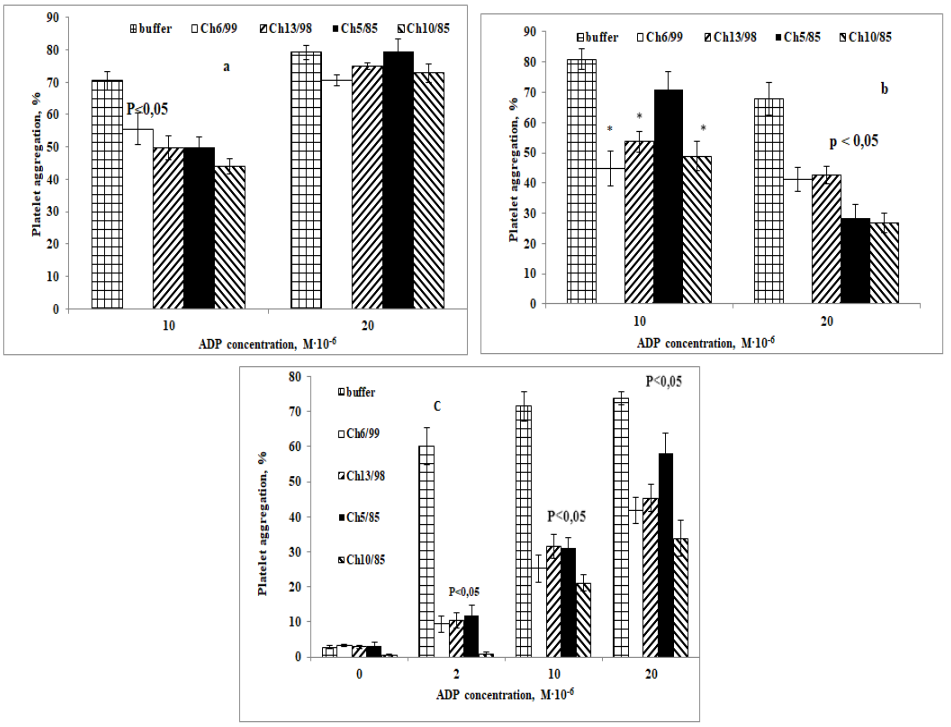

Figure 2. Influence of chitosan derivatives on the human platelet aggregation induced by ADP. Chitosan concentration: a – 0.5 mg/ml; b – 1 mg/ml; c – 2 mg/ml; * - P <0.05, the reliability of the differences with the readings in control (buffer); n = 5–18.

Fig. 2 (a, b, c) shows a significant decrease in platelet aggregation in the samples of Ch6/99, Ch13/98, Ch5/85 and Ch10/85 (at concentrations of 0.5, 1 and 2 mg/ml) induced by ADP at a concentration of $1x10^{-5}$ M. To achieve 50% inhibition at the induced platelet aggregation by ADP at a concentration of $1x10^{-5}$M, relatively equal amounts of Ch6/99, Ch13/98, Ch5/85 and Ch10/85 samples - 1.42, 1.75, 1.68 and 1.35 mg/ml, respectively, are required; when incubating plasma with samples at a concentration of 2 mg/ml, disaggregation reached 9, 12, 31 and 13%, respectively (Fig. 3).

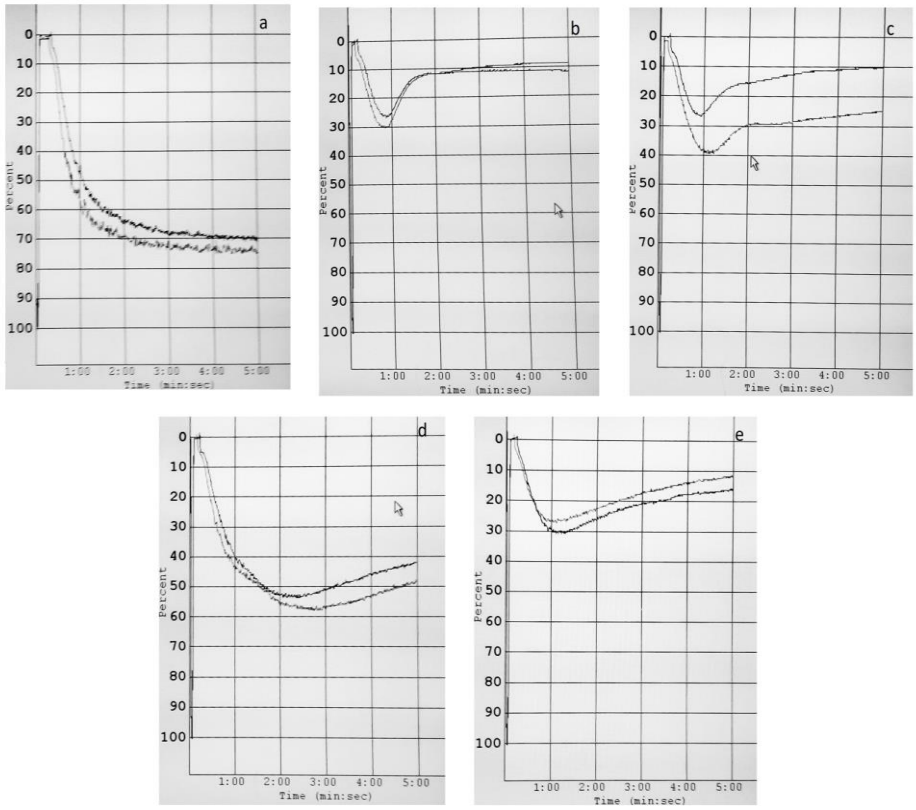

Figure 3. Photos from the computer screen with examples of aggregation curves with the induction of aggregation with ADP at a concentration of $1 \cdot 10^{-5}$ M (a – control, addition of the buffer instead of a sample; b – Ch6/99, c – Ch13/98, d – Ch5/85, e – Ch10/85; chitosan concentration of 2 mg/ml.

As the concentration of ADP was reduced to $2x10^{-6}$ M, a decrease in platelet aggregation by 6 times was observed for Ch6/99, Ch13/98, Ch5/85 samples (2 mg/ml) (Fig. 2c, Fig. 4) compared to the control; the greatest efficiency was shown by Ch10/85 – a decline to almost 0%.

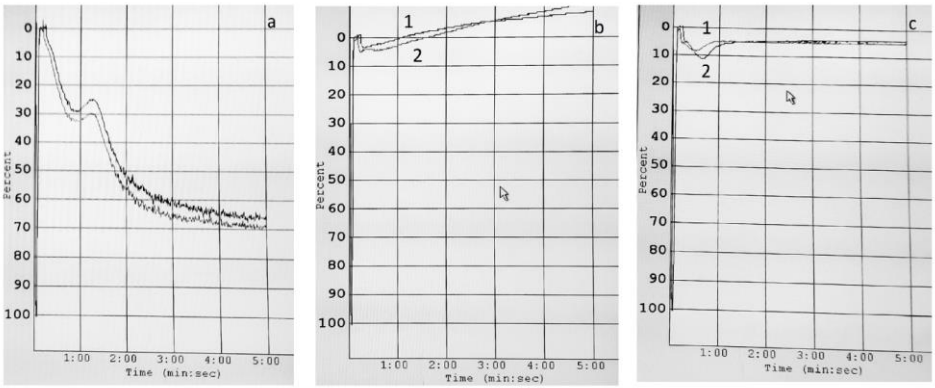

Figure 4. Photos from the computer screen with examples of aggregation curves with the induction of aggregation with ADP at a concentration of $2 \cdot 10^{-6}$ M (a – control, addition of the buffer instead of a sample; b1 – Ch6/99, b2 – Ch13/98, c1 – Ch5/85, c2 – Ch10/85; chitosan concentration of 2 mg/ml.

As the concentration of the studied sample increased, the degree of platelet aggregation (Fig. 5) induced by collagen (50 μg/ml) decreased. To achieve 50% inhibition of collagen--induced platelet aggregation (in comparison with control), 0.07, 0.09 and 1.25 mg/ml of Ch6/99, Ch13/98 and Ch10/85 samples, respectively, were required.

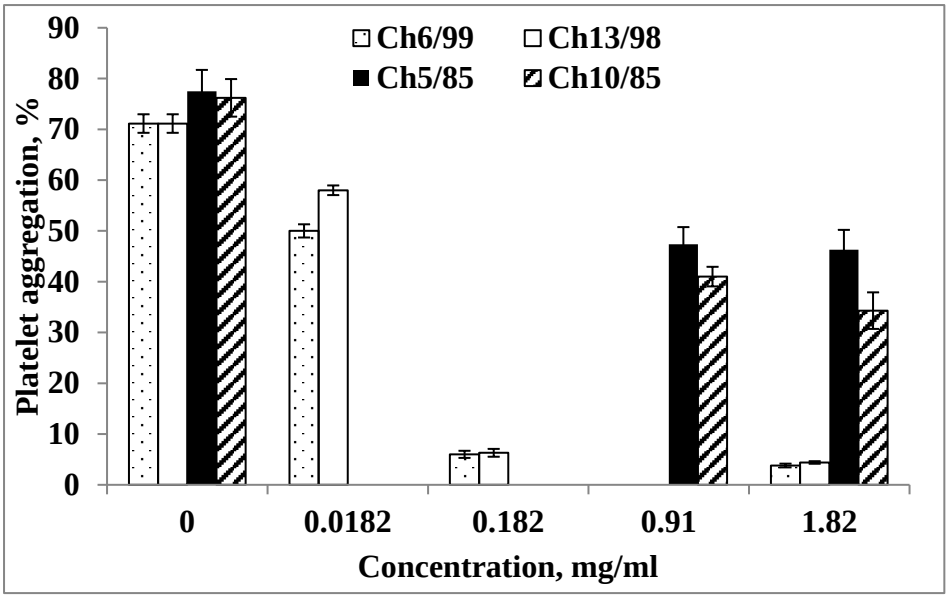

Figure 5. Influence of chitosans on human platelet aggregation induced by collagen (50 μg/ml).

Differences in the biological activity of platelets can be explained by the greater value of the positive charge of chitosan samples obtained by chemical depolymerisation.

Under the conditions used for testing biological activity (buffer pH 7.4), the protonated amino groups are conserved more in chitosan samples with a DD of 98%. The positive charge of the polymer chain of the samples of chitosan promotes interactions with the cell membrane of platelets [28]. Furthermore, both samples obtained as a result of chemical hydrolysis are characterised by greater polydispersity ─ Mw/Mn ≈2.5. Both characteristics (DD and Mw/Mn) are likely to contribute to the activity being investigated.

4. Conclusion

The studied derivatives of chitosan obtained by chemical (Ch6/99 and Ch13/98) and enzymatic (Ch5/85 and Ch10/85) depolymerisation, with almost identical MW pairs and different DD, possessed insignificant anticoagulant activity, did not provoke human platelet aggregation independently and reduced the platelet aggregation induced by ADP or collagen. At a concentration of 2 mg/ml, the studied samples reduced the platelet aggregation induced by ADP at concentrations of $2x10^{-6}$M and $1x10^{-5}$M by more than twice; the sample Ch10/85 was the most effective at the weak activation of aggregation. The most significant result indicating the specificity is that 20 times lower samples of Ch6/99 and Ch13/98 than sample Ch10/85 were required to achieve 50% inhibition of collagen-induced platelet aggregation. Higher degree of deacetylation (DD) and polydispersity (Mw/Mn) are likely to be the determining factors for the inhibition of collagen-induced platelet aggregation by Ch6/99 and Ch13/98 samples obtained by chemical depolymerization.

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