DETERMINATION OF THE HYDROLYTIC ACTIVITY OF WHOLE SALIVA USING CHITOSAN ASCORBATE AS A SUBSTRATE

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Abstract

The aim of this work was to evaluate the hydrolytic activity of whole saliva using chitosan ascorbate as a substrate. With this aim, the concentrations of N-acetyl-D-glucosamine were determined in saliva before the addition of chitosan ascorbate, directly after addition and during incubation with chitosan ascorbate for 20 hrs. In this in vitro study, sterile chitosan ascorbate was used in the form of a powder. Chitosan was obtained from krill chitin. The ratio of ascorbic acid to chitosan was 1:1. The unstimulated whole saliva showed hydrolytic activity in the presence of chitosan ascorbate as a substrate.

Key wards: *chitosan ascorbate, saliva, hydrolytic activity, N-acetyl-D-glucosamine*

Received: 14.05.2018
Accepted: 27.06.2018

1. Introduction

Saliva, being the essential environment of the oral cavity, is a very complex mixture. Whole (mixed) saliva is a solution comprising exocrine secretions from the major and minor salivary glands and non-exocrine constituents, including gingival fluid, oral epithelial cells, bacteria, and their metabolic products. Saliva is important for the execution of multiple physiological functions, such as taste, buffering, chewing, the initial digestion of some foods, swallowing, tissue hydration and lubrication, speech, maintenance of tooth integrity, protection against microbes and wound healing. Saliva contains a complex mixture of proteins with different biological roles in digestion, lubrication, and host defence [1, 2]. Saliva has a strong potential to interact with all food macronutrients because of the diversity of its enzymatic composition. Its amylolytic, proteolytic and lipolytic activities have been reported in human saliva [3, 4]. Whole saliva contains many enzymes, both exogenous i.e. of bacterial origin and endogenous. For example, a variety of hydrolases in human saliva are derived from bacteria, from the lingual glands, and from epithelial cells. [1, 2]. It is possible that some of them, like lysozyme, may take part in the hydrolysis of chitosan. Lysozyme (muramidase, N-acetylmuramylhydrolase) is an antibacterial enzyme with a concentration in human saliva that varies individually from 2 to 1000 μg/ml or more [1, 2, 5]. Temperature, pH and the concentration of NaCl in the environment have an influence on the lytic activity of lysozyme **[6]**. Lysozyme hydrolyses the β-1,4-glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine of the cell wall peptidoglycan in Gram-positive and Gram-negative bacteria [1, 2, 6]. The hydrolytic degradation of chitosan goes along with the formation of N-acetyl-D-glucosamine moieties, which play an important role in the wound healing process [7, 8, 9, 10].

Chitosan is a polysaccharide which has advantageous properties such as its non-toxicity, biocompatibility, biodegradability, antimicrobial activity, and high resistance to heat **[10, 11]**. Our earlier investigations concerned the clinical application of chitosan ascorbarte (ChA) as a multifunctional dressing that could be used in the oral cavity. The chitosan ascorbate was assessed taking into consideration its haemostatic, hygroscopic and film-forming properties as well as the degree of adhesion to soft tissues [12, 13]. We also investigated the antimicrobial activity of ChA on oral microbiota [14].

The aim of this work was to evaluate the hydrolytic activity of saliva using chitosan ascorbate as a substrate.

2. Materials and Methods

In this study, sterile chitosan ascorbate in the form of powder was used. Chitosan was obtained from krill chitin. The viscosity of chitosan was 2090 mPa·s and the degree of deacetylation was 62.77%. The ratio of ascorbic acid to chitosan was 1:1 (percentage by weight). Chitosan ascorbate was prepared in the Sea Fisheries Institute (Gdynia, Poland). The samples of saliva were taken from 30 healthy volunteers. Unstimulated mixed saliva was collected under standardised conditions: saliva collection (7 ml) was performed in the morning (9.30–10.00) two hours after breakfast. The samples of unsterilised saliva were used to prepare 30 separate experimental kits. Each of them was made up of 2 different incubation mixtures marked A and B. Control mixture A (pH 5.9) consisted of 3 ml of fresh, non-centrifuged saliva + 1 ml of buffer solution (0.6 M sodium citrate, 1.2 M Na_2HPO_4, pH 5.1), while mixture B (pH 5.8) consisted of 3 ml of fresh, non-centrifuged saliva + 20 mg chitosan ascorbate + 1 ml of buffer solution (0.6 M sodium citrate, 1.2 M Na_2HPO_4, pH 5.1).

DOI: 10.15259/PCACD.23.09

Control mixture C (pH 5.8) consisted of 20 mg chitosan ascorbate +1 ml of buffer solution (0.6 M sodium citrate, 1.2 M Na_2HPO_4, pH 5.1) + 3 ml of bi-distilled water (n = 30).

The incubation of all mixtures was carried out simultaneously at 37°C in a water bath under the cover and under constant shaking for 20 hrs. N-acetyl-D-glucosamine concentration was determined in mixture B before incubation twice: immediately before the addition of chitosan ascorbate and directly after the addition of 20 mg of chitosan ascorbate. The next determinations were carried out in mixture B with chitosan ascorbate after 1, 8 and 20 hrs of incubation. In the control mixtures A and C, the concentration of N-acetyl-D-glucosamine was determined before incubation and after 1, 8 and 20 hrs of incubation.

Each sample was analysed to detect N-acetyl-D-glucosamine according to the modified method of Reissig et al. [15] for N-acetyl-aminosugar determination. Absorbance was measured at 544 nm by Beckmann spectrophotometer and the concentration of N-acetyl-D-glucosamine was calculated in µg/ml from the standard curve.

Determinations of statistical significance were performed using Student's *t* test for dependent and independent values. For all analyses, a probability of $p<0.05$ was considered to be significant. The results are presented as mean ±SD, Me (median) and range.

3. Results and Discussion

The data in Table 1 present concentrations of N-acetyl-D-glucosamine determined in samples taken before (initial sample) and during the course of a 20-hour incubation (mixture A, B and C). Values of N-acetyl-D-glucosamine in saliva without ChA before incubation were between 0 and 6.8 µg/ml with a mean of 4.5±1.6 µg/ml (control mixture A and mixture B). A significant increase in the concentration of N-acetyl-D-glucosamine in saliva was observed in mixture B directly after the addition of ChA: values of N-acetyl-D-glucosamine were between 2.3 and 13.5 µg/ml with a mean of 6.1±3.2 µg/ml. A significant increase in the concentration of N-acetyl-D-glucosamine in mixture B was observed after 8 hrs (9.6±3.5 µg/ml) and 20 hrs (17.6±8.6 µg/ml). In the case when an experimental mixture contained saliva only (control mixture A), a significant increase in the concentration of N-acetyl-D-glucosamine was observed only after 1 hr of incubation (5.5±1.9 µg/ml). The concentrations of N-acetyl-D-glucosamine in control mixture A after 8 and 20 hrs of incubation were similar (5.4±2.5 µg/ml and 5.9±3.4 µg/ml). In the case when an experimental mixture contained ChA only (control mixture C), the initial concentration of N-acetyl-D-glucosamine was low (3.4±2.4 µg/ml). A significant increase in the concentration of N-acetyl-D-glucosamine was observed after 20 hrs of incubation (6.3±3.4 µg/ml). During incubation, the concentration of N-acetyl-D-glucosamine was always significantly higher in mixture B (saliva + buffer + ChA) in comparison with the concentration of N-acetyl-D-glucosamine confirmed in control mixture A (saliva + buffer) and control mixture C (water + buffer + ChA) (statistically significant differences).

On the basis of the results obtained from the *in vitro* study, we confirmed that human saliva showed hydrolytic activity in the presence of ChA as a substrate. The progress of ChA hydrolysis in saliva was slow. The concentration of N-acetyl-D-glucosamine in mixture B increased in the samples collected during the course of incubation. After 20 hrs, the average concentration of N-acetyl-D-glucosamine in mixture B was about three times higher than that measured in samples from mixture A (Table 1).

It is possible that saliva could also show a similar hydrolytic activity *in vivo* in the oral cavity in the presence of the ChA dressing. As mentioned above, chitosan ascorbate

Table 1. The hydrolytic activity of saliva in samples taken before incubation (initial sample) and in the course of 20-hour incubation

<table>
<tr><th rowspan="4">MIXTURE</th><th rowspan="4">No of samples</th><th colspan="12">The concentration (µg/ml) of N-acetyl-D-glucosamine</th><th rowspan="4">The ratio of N-acetyl-D-glucosamine concentrations obtained during incubation in the reference to initial samples</th></tr>
<tr><th colspan="4" rowspan="3">INITIAL SAMPLE</th><th rowspan="3">$\bar{x}$ ±SD</th><th rowspan="3">Me Range</th><th colspan="6">INCUBATION TIME</th></tr>
<tr><th colspan="2">1 hr</th><th colspan="2">8 hrs</th><th colspan="2">20 hrs</th></tr>
<tr><th>$\bar{x}$ ±SD</th><th>Me Range</th><th>$\bar{x}$ ±SD</th><th>Me Range</th><th>$\bar{x}$ ±SD</th><th>Me Range</th></tr>
<tr><td>A</td><td>30</td><td colspan="4">Saliva + Buffer</td><td>4.5[b] ±1.6</td><td>5.6 0.0 – 6.8</td><td>5.5[e] ±1.9</td><td>5.6 2.1 - 9.0</td><td>5.4[h] ±2.5</td><td>5.6 2.1 - 13.5</td><td>5.9[k] ±3.4</td><td>5.6 2.1 - 18.0</td><td>1:1.22:1.20:1.31</td></tr>
<tr><td rowspan="2">B</td><td rowspan="2">30</td><td rowspan="2">Saliva + Buffer (before ChA addition to saliva)</td><td>$\bar{x}$ ±SD</td><td>Me Range</td><td rowspan="2">Saliva + Buffer + ChA (directly after ChA addition to saliva)</td><td rowspan="2">6.1[c] ±3.2</td><td rowspan="2">5.6 2.3 - 13.5</td><td rowspan="2">6.5[f] ±2.7</td><td rowspan="2">5.6 2.8 - 13.5</td><td rowspan="2">9.6[i] ±3.5</td><td rowspan="2">8.4 4.5 - 18.0</td><td rowspan="2">17.6[l] ±8.6</td><td rowspan="2">15.4 6.8 - 54.0</td><td rowspan="2">1:1.36:1.44:2.13:3.91</td></tr>
<tr><td>4.5[a] ±1.6</td><td>5.6 0.0 – 6.8</td></tr>
<tr><td>C</td><td>30</td><td colspan="4">Water + Buffer + ChA</td><td>3.4[d] ±2.4</td><td>2.8 0.0 – 9.0</td><td>3.2[g] ±2.1</td><td>2.8 0.0 – 9.0</td><td>3.3[j] ±2.2</td><td>2.8 0.0 – 9.0</td><td>6.3[m] ±3.4</td><td>5.6 0.0 - 18.0</td><td>1:0.94:0.97:1.85</td></tr>
</table>

Values rated t-Student test for dependent values statistically different: $p<0.01$: a-c, f-i, i-l, b-e, j-m
Values rated t-Student test for independent values statistically different: $p<0.01$: b-c, c-d, e-f, f-g, h-i, i-j, k-l, l

covering the tissue is in close contact with saliva, which is a natural environment of the oral cavity. Upon control examination of the patients, we noticed that the ChA dressing had remained functionally intact in the oral cavity for several to a dozen hours, and in some cases even for two days [12, 13].

To improve the solubility and application of chitosan, studies on its degradation have attracted high attention from many researchers [7, 8, 9, 16, 17]. The degradation of chitosan can be performed by either acid or enzyme hydrolysis. Enzymatic hydrolysis is advantageous because of its mild reaction conditions, high specificity, and non-modification of sugar rings. Chitosanase is the specific enzyme for chitosan hydrolysis. Some nonspecific enzymes such as cellulases [9, 18, 19], lipases [9, 18, 19], and proteases [9, 18, 19] have the ability to hydrolyse chitosan and those were comparable with the results achieved by chitosanase. However, the disadvantage of the enzymatic hydrolysis method is, in general, its low reaction rate [9, 19]. In our study, the hydrolysis of ChA was higher in the presence of saliva (mixture B) than in buffer solution (mixture C).

After 20 hrs, the average concentration of N-acetyl-D-glucosamine in mixture B was about three times higher than that in the control mixture C (Table 1).

4. Conclusions

In the presence of ChA as a substrate, human saliva showed hydrolytic activity. The concentration of N-acetyl-D-glucosamine was increasing in the samples collected in the course of incubation. The progress of ChA hydrolysis in saliva was slow. The significant increase in the concentration of N-acetyl-D-glucosamine was observed directly after the addition of ChA to saliva and after 8 and 20 hrs of saliva incubation with ChA.

Chitosan ascorbate can be used as a substrate to the investigation of hydrolytic activity in whole saliva. The described experimental conditions make the *in vitro* process of the biodegradation of chitosan in saliva possible.

5. References

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