

Determination of lysosomal exoglycosidases in human saliva

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Background: Currently we observe a growing interest in human saliva as a non-invasive material for diagnosis and monitoring of general and oral diseases. **Methods:** The aim of our study was adaptation of the Marciniak *et al.* (Marciniak J, Zalewska A, Popko J, Zwierz K, 2006, *Clin Chem Lab Med* 44: 933–937) method for determination of HEX and GLU activity in synovial fluid, and for determination of: HEX and GLU, as well as MAN, GAL, and FUC activity in human saliva. **Results:** Under optimal conditions, 10 µl of saliva for HEX, and 30 µl for GLU, MAN, GAL and FUC, were sufficient for determination of human salivary exoglycosidases activity with variation coefficient ranging from 0.89 for GLU to 0.99 for GAL. **Conclusion:** The adapted method for exoglycosidases activity determination in human saliva is sufficiently sensitive and precise to use in clinical diagnosis.

Key words: β-galactosidase, β-glucuronidase, α-fucosidase, α-mannosidase, N-acetyl-β-D-hexosaminidase, saliva

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INTRODUCTION

Salivary exoglycosidases are a group of enzymes that are responsible for degradation of oligosaccharide chains of glycoconjugates during organogenesis, growth and normal tissue turnover. Glycoconjugates — include glycoproteins and glycolipids, constituting cellular membranes, for e.g. in salivary gland cells and oral mucosa as well as heteropolysaccharide chains of glycosaminoglycans and proteoglycans constituting extracellular matrix with glycoproteins (Zwierz *et al.*, 1989). Glycoconjugate catabolism depends on continuous renewal of cellular elements, it is started by endoglycosidases and then successively cleaved at the non-reducing end of oligosaccharide chains by exoglycosidases (Zwierz *et al.*, 1989; Winchester, 2005). Activity of exoglycosidases in saliva is normally quite low. Elevation of specific activities of salivary exoglycosidases may indicate a loss in equilibrium between degradation of older and synthesis of newest elements, which is observed in different pathological states of salivary glands (Knaś *et al.*, 2006; Waszkiewicz *et al.*, 2008; Waszkiewicz *et al.*, 2009).

There were different methods described for exoglycosidase activity determination based on different substrates, e.g. sugar derivatives of 4-nitrophenol (Knaś *et al.*, 2006), phenolphthalein (Lamster *et al.*, 1987) and 4-meth-

ylumbelliferone (Lamster *et al.*, 2003) for determination of the salivary exoglycosidases activity. We determined activity of salivary lysosomal enzymes by the method of Marciniak *et al.* (Marciniak *et al.*, 2006), based on 4-nitrophenol derivatives of appropriate sugars, specifically for synovial fluid HEX and GLU. Increasing interest in the activity of salivary exoglycosidases persuaded us to standardize a method for the determination of activity of majority of salivary exoglycosidases utilizing 4-nitrophenol derivatives of the appropriate sugars. We have previously found elevated activity of HEX and its isoenzymes in synovial fluid and serum of the patients with different arthropathies (Popko *et al.*, 2006), and selected saliva of women with rheumatoid arthritis, as the incidence of rheumatoid arthritis is three times higher in women than in men (Zalewska *et al.*, 2013).

In the current study we present kinetics and optimal conditions for measuring the activity of lysosomal FUC, GAL, GLU, HEX and MAN in human saliva, applicable for clinical diagnosis.

MATERIALS AND METHODS

Experimental. Forty rheumatoid arthritis (RA) from the Rheumatology and Internal Disease Department, Medical University in Białystok (Poland) and forty generally healthy women, matched in age and exclusion criteria to the RA group, recruited at the Department of Conservative Dentistry Medical University in Białystok, Poland, participated in the study. All RA women fulfilled the American College of Rheumatology revised criteria for RA. The study was conducted at the University Hospital in Białystok, Poland. The survey was carried out between April 2007 and September 2010. Patients were excluded if they smoked or used medication which could cause oral or eye dryness. None of the patients had diabetes mellitus or hypertension. Patients with HCV and HIV infection as well as with periodontitis or gingivitis were also excluded.

Written informed consent was obtained from each patient, after the aims and methodology of the study were explained. The study was approved by the Ethics Committee of the Medical University of Białystok (permission number R-I-002/282/2007).

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Abbreviations: HEX, N-acetyl-β-D-hexosaminidase; MAN, α-mannosidase; FUC, α-fucosidase; GAL, β-galactosidase; GLU, β-glucuronidase; RA, rheumatoid arthritis; UWS, unstimulated whole saliva

Unstimulated whole saliva (UWS) collection: Subjects were instructed to refrain from food and beverages, except water, for two hours before saliva collection. To minimize circadian influences, all salivary samples were collected between 8 a.m. and 10 a.m. During sample collection patients were protected from any stimulation. The whole saliva was collected by passive spitting into plastic container, placed on crushed ice, always assisted by the same person (A.Z). Immediately after collection, salivary samples were centrifuged at $3000 \times g$ for 20 minutes at 4°C , to remove cells and debris. The resulting supernatants were divided and transferred to plastic test tubes (Eppendorf type), frozen and kept at -80°C , until analyzed.

Enzyme substrates: As a substrate for determination of the HEX activity, 4-nitrophenyl-N-acetyl- β -glucosaminide (Sigma, St. Louis, MO, USA) was used, as well as 4-nitrophenyl- β -D-glucuronide (Fluka Chemie; Sigma, St. Louis, MO, USA) for β -GLU and 4-nitrophenyl- β -D-galactopyranoside (Sigma) for β -GAL, 4-nitrophenyl- α -D-fucopyranoside (Sigma) for α -FUC and 4-nitrophenyl- α -D-mannopyranoside for

α -MAN (Sigma). The calibrant, 4-nitrophenol (Sigma), was prepared at concentration of 0.3 mM in 100 mM citrate-phosphate buffer, pH 4.7.

Instrumentation. Spectrophotometric measurements were carried out using a MR-96 A microplate reader (Mindray, Shenzhen, ChRL) at 405 nm, and immunosorbent assays plates of 96 wells (MaxiSorp; Nunc Intermed, Copenhagen, Denmark).

Statistical analysis. Statistical analysis was performed with Statistica 9.0 using *t*-Student test (StatSoft, Cracov, Poland). Results were presented as mean and S.D.

RESULTS

Calibration

Activity of HEX, GAL, MAN, FUC and GLU in the samples was analyzed using a calibration curve for 4-nitrophenol. Calibration curve was obtained by adding 200 mM borate buffer, pH 9.8 to standard solution of 4-nitrophenol, and intensity of the yellow color produced was measured at 405 nm with a microplate reader.

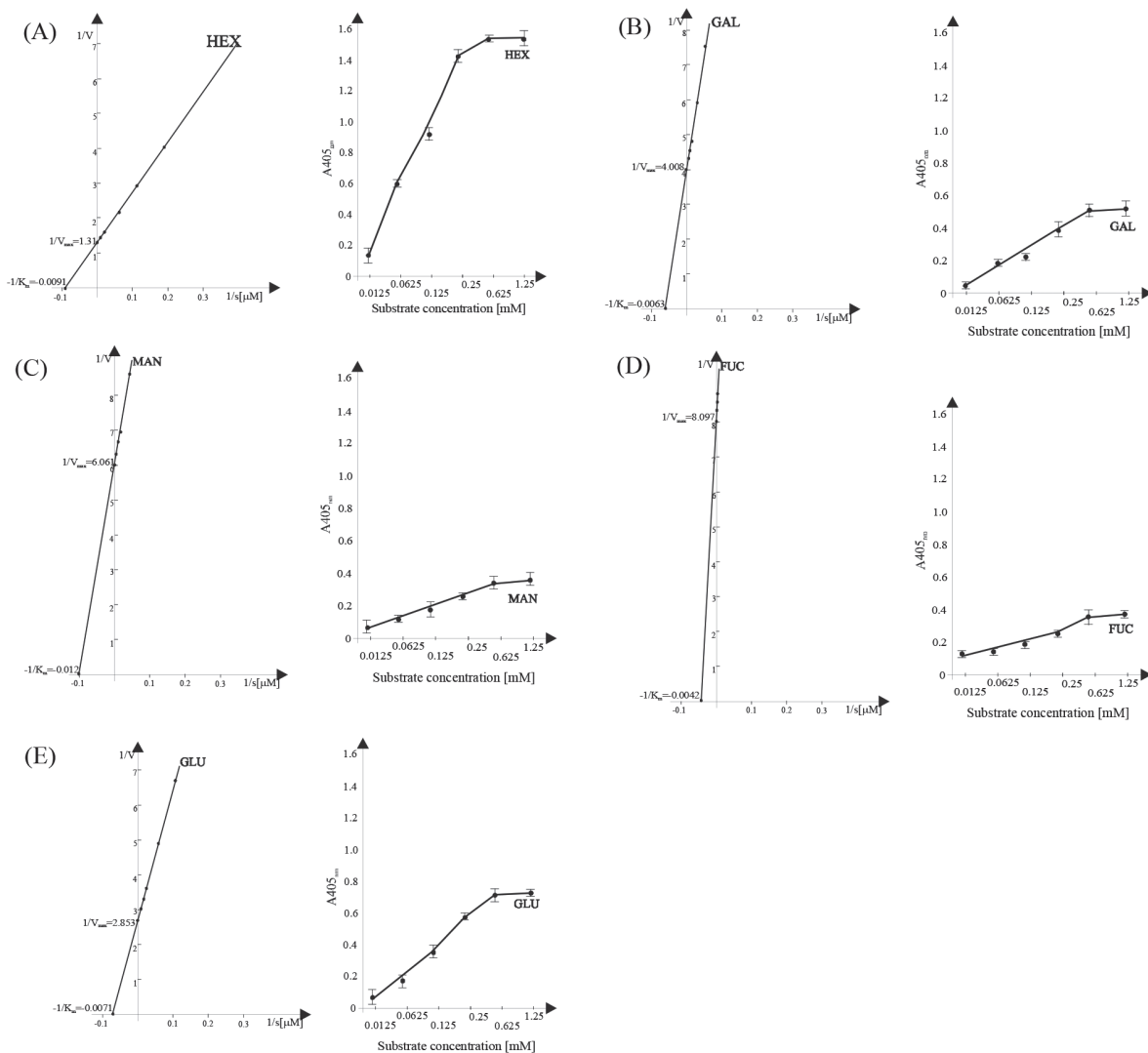


Figure 1. Lineweaver-Burk and Michaelis-Menten plots for determination of K_m for HEX, GLU, GAL, MAN and FUC (mean values of 40 measurements \pm S.D.).

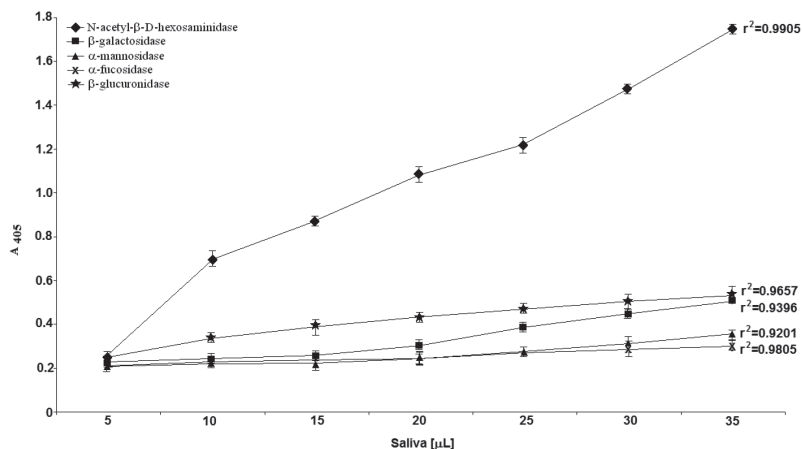


Figure 2. Effect of enzyme amount on HEX, GLU, GAL, MAN and FUC activities (mean values of 40 measurements \pm S.D.).

Substrate concentration

For the determination of salivary exoglycosidases K_m , to 10 μ L of UWS for HEX and 30 μ L of UWS for GAL, GLU, MAN, and FUC, 30 μ L of substrate was added at the concentrations indicated at Fig. 1A–1E, and 40 μ L of 100 mM citrate-phosphate buffer pH 4.7 for HEX, pH 4.3 for GAL, MAN, FUC and 200 mM sodium acetate buffer pH 4.5, for GLU. Microplates were incubated for 60 min at 37°C with constant shaking. Reactions were stopped by adding 200 μ L of 200 mM borate buffer, pH 9.8. The K_m value calculated was: 0.11 mM for HEX (Fig. 1A), 0.16 mM for GAL (Fig. 1B) for GAL, 0.08 mM for MAN (Fig. 1C), 0.23 mM for FUC (Fig. 1D), 0.14 mM for GLU (Fig. 1E).

Effect of the amount of saliva in the incubation mixture: To evaluate influence of the amount of saliva on activity of salivary exoglycosidases, volumes of 5–30 μ L of UWS, 30 μ L of substrate at concentration 1.1 mM (HEX), 1.6 mM (GAL), 1.4 mM (GLU), 0.8 mM (MAN), 2.3 mM (FUC) and appropriate amount of 100 mM citrate-phosphate buffer pH 4.7 (HEX), pH 4.3 (FUC, GAL, MAN) or 200 mM sodium acetate buffer pH 4.5 (GLU), to obtain 100 μ L of total fluid in the well, were added to microplate wells. Microplates were incubated for 60 min at 37°C, with constant shaking, and reactions were stopped by adding 200 μ L of 200 mM borate buffer, pH 9.8. Relation between amount of saliva and $A_{405\text{ nm}}$ of the released 4-nitrophenol by particular exoglycosidase is presented in Fig. 2.

Effect of pH on the enzyme activity: Salivary exoglycosidase activity was examined at pH 3.5–5.5 at a substrate concentration of 1.1 mM (HEX), 1.6 mM (GAL), 0.8 mM (MAN), 2.3 mM (FUC) and 1.4 mM (GLU) (Fig. 3). Exoglycosidase activity was determined as described above. Majority of lysosomal enzymes demonstrate optimal activity in a slightly acidic environment, and their activity diminishes at neutral pH. We demonstrated that HEX, the most active of exoglycosidases (Zwierz *et al.*, 1989), has optimum at pH 4.7 (Fig. 3A), GLU at pH 4.5 (Fig. 3A), and GAL, MAN, FUC at pH 4.3 (Fig. 3B), respectively.

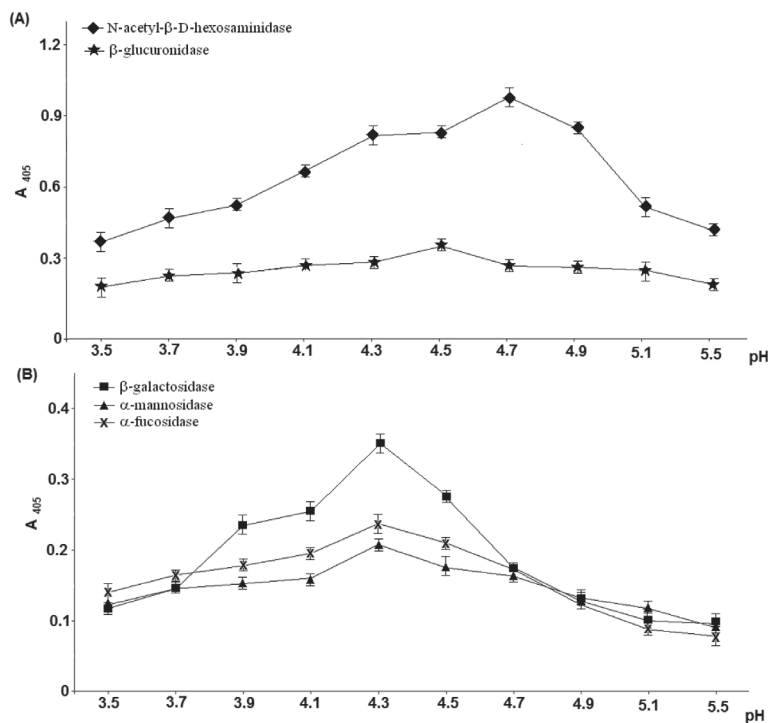


Figure 3. Effect of pH on: (A) HEX, GLU, (B) GAL, MAN and FUC activities (mean values of 40 measurements \pm S.D.).

Incubation time

To establish optimal incubation time we used concentrations of substrates as in Fig. 4, and 10 μ L of UWS for HEX and 30 μ L of UWS for the remaining exoglycosidases. Samples were incubated between 0 and 120 min (Fig. 4).

Determination of exoglycosidase activity in human saliva: We propose the following procedure for determination of salivary FUC, GAL, GLU, HEX and MAN activity in 96 wells assays plates.

Into each microplate well, 10 μ L of saliva for HEX and 30 μ L of saliva for FUC, GAL, MAN and GLU was added, as well as 30 μ L of substrate at concentration: 1.1 mM (HEX), 1.6 mM (GAL), 1.4 mM (GLU), 0.8 mM (MAN) and 2.3 mM (FUC), as well as 40 μ L of 100 mM citrate-phosphate buffer pH 4.7 (HEX), pH 4.3 (GAL, MAN, FUC) and sodium acetate buffer pH 4.5 (GLU). Unless otherwise indicated, incubation was carried out for 60 min at 37°C, with

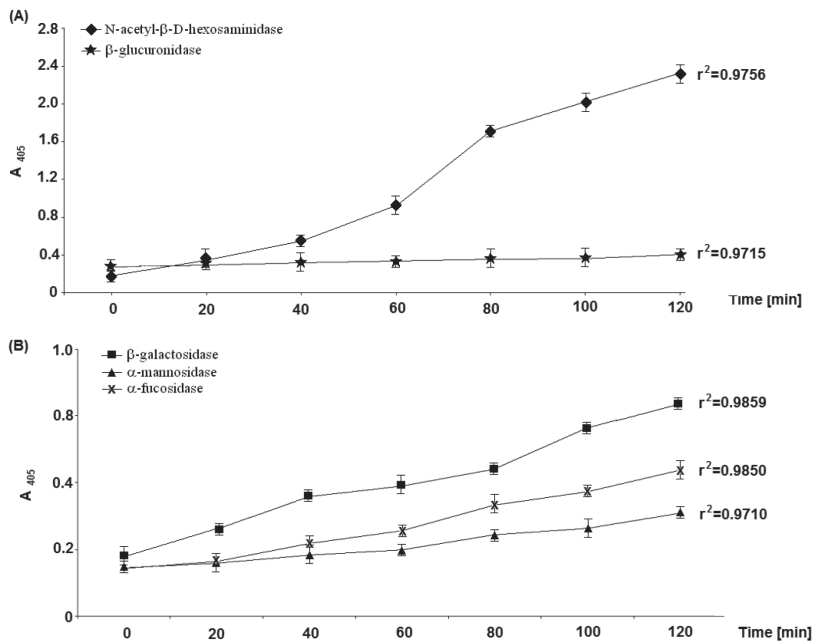


Figure 4. Effect of incubation time on (A) HEX, GLU, (B) GAL, MAN and FUC activities (mean values of 40 measurements \pm S.D.).

constant shaking; the reaction was stopped by adding 200 μ L of 200mM borate buffer pH 9.8. The A 405 nm was determined with Elx800™ microplate reader (MR-96 A, Mindray).

Precision

Intra-assay variability was assayed using human saliva from a patient with RA. The activity was measured 20 times in each sample. Activity and standard deviation was estimated to be: 16.8 pKat/mL \pm 0.61 for HEX, 2.53 pKat/mL \pm 0.4 for GAL, 2.78 pKat/mL \pm 0.66 for MAN, 1.56 pKat/mL \pm 0.31 for FUC and 1.24 pKat/mL \pm 0.36 for GLU. The coefficient of variation was: 0.932% for HEX, 0.99% for GAL, 0.989% for MAN, 0.956% for FUC and 0.89% for GLU.

RA *versus* the reference group: In the saliva of RA patients activities of all of the examined exoglycosidases were significantly higher in comparison to the reference group (Fig. 5).

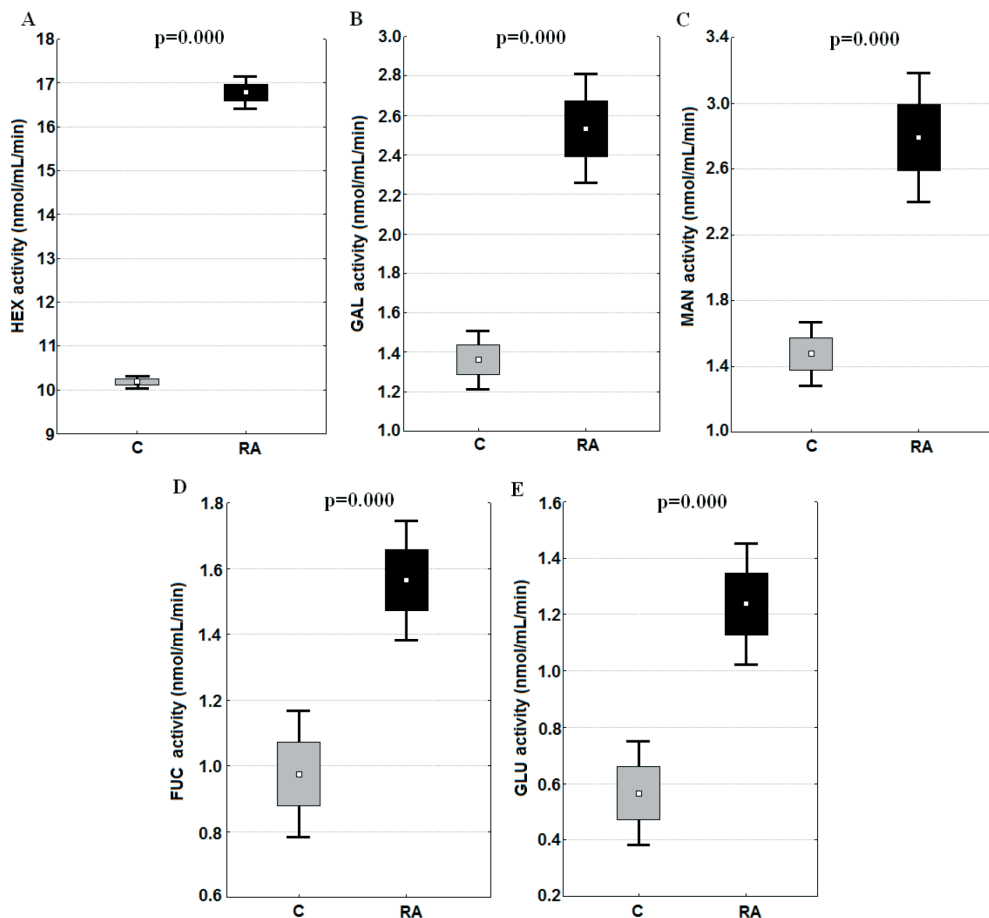


Figure 5. Activities of salivary exoglycosidases of 40 RA and 40 healthy women. C — control; RA — rheumatoid arthritis. A — HEX, B — GAL, C — MAN, D — FUC, E — GLU.

DISCUSSION

It was reported that lysosomal exoglycosidases take part in homeostasis of oral cavity and changes in their activities reflect oral health. The first reports on salivary exoglycosidases were focused mainly on association of salivary exoglycosidases and periodontal disease. Nakamura and Slots (Nakamura & Slots, 1983) showed that severity of the periodontal disease positively correlated with the increase in activities of lysosomal HEX, α - and β -GAL, α - and β -GLU, α - and β -glucosidase as well as α - and β -MAN. Zambon *et al.* (Zambon *et al.*, 1985) claimed that the determination of the activities of salivary β -GAL, β -GLU and α -glucosidases might be helpful in monitoring periodontal status after successful surgical treatment.

Recently, more attention is paid to determination of salivary exoglycosidases activity in diagnostics of general diseases. It was also shown that changes in the salivary exoglycosidases activities may be related to changes in the ultrastructure and function of the salivary glands. Bierć *et al.* (Bierć *et al.*, 2010) reported significant increase in activities of GLU, GAL, and HEX and its isoenzymes, in salivary gland tissue of patients with salivary glands tumors. Authors hypothesized that salivary exoglycosidases may take part in tumor development and metastasis. Knaś *et al.* (Knaś *et al.*, 2006) reported that combination of diabetes and smoking gives significant increase in the specific activity of salivary HEX and its isoenzymes. Waszkiewicz *et al.* (Waszkiewicz *et al.*, 2008) suggested that significant increase in salivary HEX and its isoenzymes activity after large single dose of ethanol, can be a marker of the harmful level of drinking, which is damaging salivary glands and/or oral mucosa. Authors claimed that since the activity of isoenzyme A reflects the secretory status of the cell (Waszkiewicz *et al.*, 2013), the significant increase of HEX A activity in saliva, suggests that increased production of HEX A is related to functional changes in salivary glands cells after harmful drinking. The activity of HEX B is an outcome of breakdown of the cell membrane (Waszkiewicz *et al.*, 2013), so significant increase in HEX B specific activity in saliva may be an indicator of advanced pathological changes in the structure of the salivary glands cells. Results of the present study show that increase in the catabolism of glycoconjugates of the salivary gland connective tissue (expressed as elevated activity of the salivary exoglycosidases) is more pronounced in the case of RA patients in comparison to the reference group. These changes in salivary exoglycosidases activities may be related to changes in the ultrastructure and function of the salivary glands.

As saliva is more and more frequently used as a non-invasive diagnostic material, we decided to standardize determination of five salivary exoglycosidases activities utilizing 4-nitrophenol derivatives of appropriate sugars, by adaptation to saliva of the Marciniak *et al.*, (Marciniak *et al.*, 2006) method for exoglycosidases determination in synovial fluid. Since assays for enzymes activity determination are recommended to be carried out at a substrate concentration that is approximately 10 times higher than K_m of the enzyme (Dixon *et al.*, 1979), we determined K_m to be: 0.11 mM for HEX, 0.16 mM for GAL, 0.08 mM for MAN, 0.23 mM for FUC and 0.14 mM for GLU. In the routine determination of salivary exoglycosidase activity we use the substrate concentration for all enzymes that is 10 times higher than their K_m .

Our results are in agreement with previously published papers that majority of lysosomal exoglycosidases are ac-

tive at slightly acidic pH, and HEX is the most active of exoglycosidases in tissues (Zwierz *et al.*, 1999; Popko *et al.*, 2008). Optimal pH of salivary exoglycosidases was ranging from 4.3 for GAL, MAN, FUC, through 4.5 for GLU, to 4.7 for HEX.

We are presenting proof that 10 μ l of saliva is sufficient for the determination of salivary activity of HEX, and 30 μ l of saliva is appropriate for determination of activities of the remaining exoglycosidases.

CONCLUSION

Adapted method for determination of activity of lysosomal exoglycosidases in human saliva is sufficiently sensitive and precise to use in clinical diagnosis of salivary glands and oral health.

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Conflict of interest

We declare that there are no conflicts of interest, funding, employment or leadership and honorarium.

REFERENCES

- Bierć M, Minarowski Ł, Woźniak Ł, Chojnowska S, Knaś M, Szajda SD *et al.* (2010) The activity of selected glycosidases in salivary gland tumors. *Folia Histochem Cytobiol* **48**: 471–474.
- Dixon M, Webb EC, Thorne JC, Tipton KF (1979) *Enzymes*. Longman, London.
- Knaś M, Karaszewska K, Szajda SD, Zarzycki W, Dudzik D, Zwierz K (2006) Saliva of patients with Type I diabetes: effect of smoking on activity of lysosomal exoglycosidases. *Oral Dis* **12**: 278–282.
- Lamster IB, Harper DS, Fiorello LA, Oshrain RL, Celenti RS, Gordon JM (1987) Lysosomal and cytoplasmic enzyme activity, crevicular fluid volume, and clinical parameters characterizing gingival status and shallow to intermediate probing depths. *J. Periodontol* **58**: 614–621.
- Lamster IB, Kaufman E, Grbic JT, Winston LJ, Singer RE (2003) β -glucuronidase activity in saliva: relationship to clinical periodontal parameters. *J Periodontol* **74**: 353–359.
- Marciniak J, Zalewska A, Popko J, Zwierz K (2006) Optimization of an enzymatic method for the determination of lysosomal N-acetyl-beta-D-hexosaminidase and beta-glucuronidase in synovial fluid. *Clin Chem Lab Med* **44**: 933–937.
- Nakamura M, Slots J (1983) Salivary enzymes. Origin and relationship to periodontal disease. *J Periodontol Res* **16**: 559–569.
- Popko J, Marciniak J, Ilendo E, Knaś M, Guszczyn T, Stasiak-Barmuta A *et al.* (2008) Profile of exoglycosidases in synovial cell cultures derived from human synovial membrane. *Cell Biochem Biophys* **51**: 89–95.
- Popko J, Marciniak J, Zalewska A, Górska A, Zwierz K, Sierakowski S, Urban M (2006) Activity of N-acetyl-beta-hexosaminidase and its isoenzymes in serum and synovial fluid from patients with different arthropathies. *Clin Exp Rheumatol* **24**: 690–693.
- Waszkiewicz N, Chojnowska S, Zalewska A, Zwierz K, Szulc A, Szajda SD (2013) Salivary hexosaminidase in smoking alcoholics with bad periodontal and dental states. *Drug Alcohol Dependence* **129**: 33–40.
- Waszkiewicz N, Szajda SD, Jankowska A, Kępka A, Dobryniowski J, Szulc A *et al.* (2008) The effect of the binge drinking session on the activity of salivary, serum, urinary beta hexosaminidase: Preliminary data. *Alcohol and Alcoholism* **43**: 446–450.
- Waszkiewicz N, Szajda SD, Jankowska A, Waszkiewicz M, Kępka A, Konarzewska B *et al.* (2009) Catabolism of salivary glycoconjugates in acute ethanol intoxication. *Med Sci Monit* **15**: 413–417.
- Winchester B (2005) Lysosomal metabolism of glycoproteins. *Glycobiology* **15**: 1R–15R.
- Zalewska A, Knaś M, Waszkiewicz N, Waszkiel D, Sierakowski S, Zwierz K (2013) Rheumatoid arthritis patients with xerostomia have reduced production of key salivary constituents. *Oral Surg Oral Med Oral Pathol Oral Radiol* **115**: 483–490.

Zambon J, Nakamura M, Slots J (1985) Effect of periodontal therapy on salivary enzymatic activity. *J Periodontol Res* **20**: 652–659.

Zwierz K, Gindziński A, Ostrowska L, Stankiewicz-Choroszczucha B (1989) Metabolism of glycoconjugates in human gastric mucosa — a review. *Acta Med Hung* **46**: 275–288.

Zwierz K, Zalewska A, Zoch-Zwierz W (1999) Isoenzymes of N-acetyl-beta-hexosaminidase. *Acta Biochim Polon* **46**: 739–757.