The choice of conditions for cathepsin D activity determination in human saliva

Roszkowska-Jakimiec W[1]*, Dąbrowska E[2], Gacko M[3], Karwowska A[1], Chlabicz M[3]

[1] Department of Instrumental Analysis, Medical University of Białystok, Poland
[2] Department of Social and Preventive Dentistry, Medical University of Białystok, Poland
[3] Department of Vascular Surgery and Transplantology, Medical University of Białystok, Poland

Abstract

The aim of the study was the demonstration and choice of conditions for the determination of cathepsin D activity in human mixed saliva. The 6% solution of hemoglobin, denatured with hydrochloric acid, was used as the substrate. The ratio of saliva volume to hemoglobin was 4:1 w/v. The reaction was interrupted by adding 10% trichloroacetic acid, after 6 hours of incubation at 37°C. The increase in degradation products was determined with the use of Folina and Ciocalteau method with copper modification.

Key words: human saliva, cathepsin D, method of determination, inhibitor from Vicia sativa L.

Introduction

Cathepsin D (EC 3.4.23.5) is an aspartyl endopeptidase [10]. It splits the bindings of carboxyl groups of hydrophobic aminoacid residues. It is a lysosomal enzyme acting in the acidic environment. It also occurs in small amounts in the systemic fluids, secretions, and excrements [8].

Cathepsin D activity is usually determined using protein substrates: hemoglobin, casein, and albumin [9].

* CORRESPONDING AUTHOR:
Wiesława Roszkowska-Jakimiec
Department of Instrumental Analysis
Medical University of Białystok
ul. Mickiewicza 2C, 15-089 Białystok, Poland
Tel. +48 085 748 56 00
e-mail: analist@amb.edu.pl

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Material and methods

Dithiothreitol (Serva, Germany); hemoglobin (Difco Laboratories, USA); cathepsin D inhibitor in common vetch seed coats [4]; 2,4,6-trinitrobenzensulfonic acid and ninhydrin reagent (Sigma, USA); Folin-Ciocalteau reagent (Merck Germany). Other reagents POCh, Gliwice.

The mixed saliva was collected in fasting state from 12 adults (6 women and 6 men), it was not centrifuged and stored in -75°C. Before the examination, particular saliva samples were mixed with the use of a flow homogenizer. The content of proteins in the saliva was mean 3.0 mg/ml and pH was 6.4.

1. The evaluation of hemoglobin degradation products by salivary cathepsin D

The amount of 0.2 ml of 6% hemoglobin was added to 0.8 ml of saliva. Both hemoglobin and saliva pH was 3.5. The reaction was interrupted after 6 hours of incubation in 37°C by adding 1.0 ml of 10% trichloroacetic acid (TCA). The samples precipitated at time 0 were considered to be the controls. The following were determined in supernatant fluid (obtained by centrifugation – 10000 x g for 10 min) containing hemoglobin degradation products:

- Tyrosine and peptide bindings, using Folina and Ciocalteau method with copper modification (3)
- Tyrosine using Folina and Ciocalteau method (3)
- Alpha-amine groups using ninhydrin method (2)
- Alpha-amine groups using TNBS (1)
- Aromatic aminoacids by absorbance measurement, at 280 nm of the wave length.

2. The determination of optimal pH for cathepsin D activity

The amount of 0.1 ml of 6% hemoglobin was added to 0.4 ml of saliva (pH varied from 2.5 to 5.0 with the divisions every 0.5 of pH unit) and incubated at 37°C for 6 hours. The reaction was interrupted by adding 0.5 ml of 10% TCA. The assays precipitated at time 0 were the controls. Tyrosine and peptide bindings were determined in supernatant fluid (obtained by centrifuga-

Table 1. **The content of hemoglobin degradation products by salivary cathepsin D determined with various methods**

Products	Reagent/method	Value
Tyrosine, peptide bindings	Folina and Ciocalteau, reagent copper	248.1 ±25.0 Tyr, nmol/ml
Tyrosine	Folina i Ciocalteau	123.8 ±11.4 Tyr, nmol/ml
Alpha-amine groups	ninhydrin reagent	1.6 ±0.2 Leu, μmol/ml
Alpha-amine groups	TNBS	1.2 ±0.2 Leu, μmol/ml
Aromatic aminoacids	absorbance, 280 nm	0.632 ±0.05 $E_{280\ nm}$

Table 3. **The activity of human salivary cathepsin D estimated at various time, at 3.5 pH**

Incubation time, h	Cathepsin D activity	
	Tyr, nmol/ml	%
3	98.4 ±10.2	41.2
6	238.6 ±22.6	100.0
9	310.0 ±29.6	130.0
12	364.8 ±38.2	152.9
15	398.6 ±41.8	167.0
18	458.0 ±44.8	191.9
21	498.4 ±52.0	208.9
24	526.4 ±54.2	220.6

Table 5. **The influence of dithiothreitolu and the inhibitor of common vetch seed coats on the activity of salivary cathepsin D estimated at 3.5 pH**

Compound	Cathepsin D activity	
	Tyr nmol/ml	%
Control	182.4 ±19.2	100.0
Dithiothreitol	168.6 ±17.8	92.4
Inhibitor in common vetach seed coats	66.8 ±9.8	36.6

Table 2. **The activity of human salivary cathepsin D estimated at various pHs, during 6 hours of incubation**

pH	Cathepsin D activity	
	Tyr, nmol/ml	%
2.5	18.0 ±2.3	7.5
3.0	158.6 ±12.6	65.9
3.5	240.8 ±24.5	100.0
4.0	182.0 ±16.0	75.6
4.5	98.6 ±9.8	40.9
5.0	64.3 ±7.4	26.7

Table 4. **The activity of human salivary cathepsin D estimated at various temperatures, at 3.5 pH**

Temperature, °C	Cathepsin D activity	
	Tyr nmol/ml	%
0	0.0 ±0.0	0.0
5	0.8 ±0.1	0.3
10	4.6 ±0.5	1.9
15	16.2 ±2.3	6.8
20	94.8 ±9.6	39.7
25	160.5 ±15.2	67.0
30	182.2 ±19.3	76.0
35	239.6 ±19.8	100.0
40	236.4 ±23.0	98.7
45	140.0 ±14.2	58.4
50	46.3 ±5.2	19.3
55	12.4 ±1.4	5.2
60	0.0 ±0.0	0.0

tion – 10000 x g for 10 min) using Folina and Ciocalteau method with copper modification.

3. The determination of optimal incubation time

The amount of 0.1 ml of 6% hemoglobin was added to 0.4 ml of saliva and incubated for 3, 6, 9, 12, 15, 18, and 24 hours. Reagent pH was 3.5. The procedure was completed as in point 2.

4. The choice of incubation temperature

The amount of 0.1 ml of 6% hemoglobin was added to 0.4 ml of saliva. The mixture was incubated at 0°C to 60°C, with divisions every 5°C. The procedure was completed as in point 2.

5. The influence of dithiothreitol on cathepsin D activity

The amount of 0.1 ml of ditiotreitol (5 μmol/ml) was added to 0.3 ml of saliva and preincubated for 30 min at 37°C. Then, 0.1 ml of 6% hemoglobin was added and the procedure was completed as in point 2.

6. The influence of common vetch seed coat inhibitor on cathepsin D activity

The amount of 0.1 ml of the inhibitor (25 μmol/ml) was added to 0.3 ml of saliva and preincubated for 30 min at 37°C. Afterwards, 0.1 ml of 6% hemoglobin was added and the procedure was completed as in point 2.

Results and discussion

The products of hemoglobin degradation by cathepsin D can be determined with different methods, as it can be seen in *Tab. 1*. The results showed that the most preferable method was tyrosine and peptide bindings determination using Folina and Ciocalteau reagents and with copper reagent.

Human saliva cathepsin D presented the highest activity in reaction with hemoglobin at pH 3.5 (*Tab. 2*). As *Tab. 3* showed, the optimal incubation time was 6 hours. The highest activity of the enzyme was revealed at 35-40°C (*Tab. 4*). Dithiothreitol did not affect the salivary cathepsin D activity but the inhibitor of common vetch seed coat suppressed the enzyme activity (*Tab. 5*).

The differences of cathepsin D activities were not observed as far as the sex was concerned. The saliva used for determining the cathepsin D activity should not be centrifuged but homogenized.

The results point to the fact that cathepsin D activity in the saliva, in the oral cavity, is relatively low and its determination

required many hours of incubation and lowering salivary pH to 3.5-4.0. Thus, salivary cathepsin D does not take part in protein digestion. However, we cannot exclude its participation in organic proteolysis, i.e. splitting only single peptide bindings, which results in manifestation or loss of biological properties of a given protein [7].

In inflammatory conditions, such as sialitis, gingivitis, and oral mucositis, a significant increase in cathepsin D activity, pH decrease, and local destructive activity of cathepsin D (specifically in case of tooth pocket inflammation) can occur, which is manifested by numerous enzyme activity increase [5,6].

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