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# Hsp70 is present in human saliva

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
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## Summary

**Background:** There is increasing evidence that chaperones are also present outside the cell, exerting cytokine-like effects and influencing immune recognition. Hsp70 has been found to be present in human blood sera. Chaperonins Cpn10 and Cpn60 are present in pancreatic juice, but Hsp70 is not. These observations raise the possibility that molecular chaperones may be present in other secretory fluids, such as human saliva.

**Material/Methods:** Human whole saliva was collected from six participants under resting conditions and secretory stimulation. The samples were precleared by centrifugation and sterile filtered. Salivary volume, protein concentration and amylase activity were determined. For detection of Hsp70 saliva proteins were separated on a 12.5% SDS-PAGE. Semi-dry Western blot analysis was used with a primary antibody against the inducible form of Hsp70. Hsp70 bands were detected with a horseradish peroxidase-linked secondary antibody and ECL-Western blotting analysis.

**Results:** A single band was recognized around 70 kDa in the saliva of all the participants. There was a significant decrease of Hsp70, and a non-significant decrease of total protein concentration during stimulation, whereas the activity of salivary amylase increased significantly. Stimulation significantly increased the Hsp70, total protein and amylase outputs as well as the amylase/protein ratio, and decreased the Hsp70/amylase and Hsp70/protein ratios.

**Conclusions:** Hsp70 is secreted to saliva, but unlike amylase is not transported by the exocytotic secretory mechanisms of acinar cells. Passive transport mechanisms of Hsp70 from blood serum or from salivary gland cells may be major routes of salivary Hsp70 secretion.

**key words:** **molecular chaperone • Hsp70 • saliva • secretoric stimulation • amylase • protein secretion**

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## BACKGROUND

Hsp70 is an important member of the family of molecular chaperones [1]. The expression of Hsp70 is stress-induced; however, it is present in substantial amounts at basal levels (constitutive, 73 kDa form), and is up-regulated in response to stress (inducible, 72 kDa form). These proteins are usually considered to be intracellular, preventing protein aggregation and refolding damaged proteins. However, there is increasing evidence that chaperones are also present outside the cell, exerting cytokine-like effects and influencing immune recognition [2]. There is important evidence pointing to the extracellular action of Hsp70:

– Hsp70 has been found in human blood sera [3,4],
– administration of exogenous Hsp70 to human promonocytic cells prior to treatment with tumor necrosis factor-alpha significantly lowers the number of apoptotic and necrotic cells (through cell surface interaction followed by internalisation) [5],
– exogenous Hsp70 administration protects arterial smooth muscle cells from toxic effects through association with the cell surface (in this case without internalization) [6].

All these data strongly indicate that extracellular Hsp70 may play an important role in preventing cell damage, or in refolding damaged proteins outside the cell. It has also been reported that the chaperonins Cpn10 and Cpn60 are present in the pancreatic juice, but Hsp is not [7]. These observations have raised the possibility that molecular chaperones may be present not only in the pancreatic juice, but also in other secretory fluids, such as human whole saliva, which would point to the possible role of chaperones in maintaining, saving, and repairing the integrity of the mucosal surfaces of the oral cavity and upper gastro-intestinal tract, through extracellular action. In our study we provide the first demonstration of the presence of a molecular chaperone (Hsp70) in human whole saliva. We also investigated the changes in Hsp70 levels compared to the changes of salivary amylase activity and salivary total protein concentrations after secretory stimulation, in order to collect information about possible secretory mechanisms.

## MATERIAL AND METHODS

Six young healthy individuals (3 women and 3 men, aged between 22 and 31 years) took part in the study. The participants were not taking any medications or contraceptives. There were no detectable oral inflammations, parodontal inflammations, parodontal pockets, caries, or dental plaque in their mouths, which reduced the possibility of direct contamination of the collected saliva with blood to a minimum.

Secretory stimulation was done in two phases, during each of which the participants were instructed to chew a mint chewing gum for 2 minutes. Resting saliva was collected before, during, and after the stimulation phases for 5 minutes. The experiments were repeated three times, on different days, in the morning after 1 hour of fasting.

Whole saliva was collected using the method described by Schwartz *et al*. [8]. The participants were told to swallow first, and then allow the resting and stimulated saliva to accumulate in their mouth for 2 or 5 minutes, respectively, and finally to transfer it into a collecting vessel. The samples were precleared by centrifugation (20.000 x g, 4°C, 10 minutes), and sterile filtered using Millex-GV 0.22 mm pore size filters (Millipore). All experiments were repeated three times.

Salivary volume was measured with a small measuring tube. Salivary protein concentration was determined by the Lowry method [9] (Bio-Rad). Amylase activity was determined with the Phadebas Amylase Test (Pharmacia & Upjohn).

In order to detect Hsp70, 20 ml of saliva was solubilized in Laemmli sample buffer [10] and separated on a 12.5% SDS-PAGE gel using a Bio-Rad slab mini-gel system. Low-flow-hypoxic rat liver homogenate [11] was used as a positive control for Hsp70. The rat liver was removed surgically and perfused via a cannula inserted into the portal vein with Krebs-Henseleit bicarbonate buffer [pH 7.4, 37°C] saturated with a 95% $O_2$ – 5% $CO_2$ mixture in a non-recirculating system at flow rates of ~1 ml/g/min for 80 min. The liver tissue was homogenized with a buffer containing 10 mmol/L of Tris-HCl, pH 7.4, 0.25 mol/L of sucrose, and 0.1 mmol/L of EDTA. An amount of liver homogenate containing 9 mg protein solubilised in Laemmli sample buffer was used as a positive control. A semi-dry Western blot analysis on nitro-cellulose membrane was used to detect Hsp70, with a primary antibody against the inducible (72 kDa) form of Hsp70 (StressGen). Hsp70 bands were detected with a horseradish peroxidase-linked secondary antibody against mouse IgG (Amersham), and with the ECL-Western blotting analysis system (Amersham). 3% blotting grade blocker non-fat dry milk (Bio-Rad) was used for blocking after the primary antibody at 25°C for 1 hour. X-ray photographs were scanned (Epson Perfection 1640SU), and Image Master TotalLab software (version 1.11) was used for quantitative analysis of the detected bands.

### Statistical analysis

For purposes of statistical evaluation the Statistical Package for the Social Sciences SPSS/PC version 8.0 was used [12]. Student's t-test for two samples was used; the similarities of deviations were checked with the F-test. We considered differences to be significant at the level of $p \geq 0.05$.

Our experiments were planned and carried through in compliance with the Helsinki Declaration of the World Medical Association [13]. Only adult subjects took part, voluntarily, knowing all of the important circumstances and parameters in the study. Sample collection was completely noninvasive.

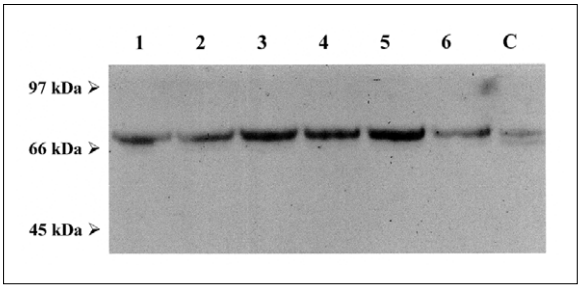


**Figure 1.** Detection of Hsp70 in whole human saliva. 20 ml of first control (resting) saliva of each participant (lanes 1 through 6) was analyzed by SDS-PAGE and Western blot analysis as described in Materials and Methods. C: positive control from hypoxic liver. Bands are representative from 3 experiments (females: lanes 1-3, males: 4-6)

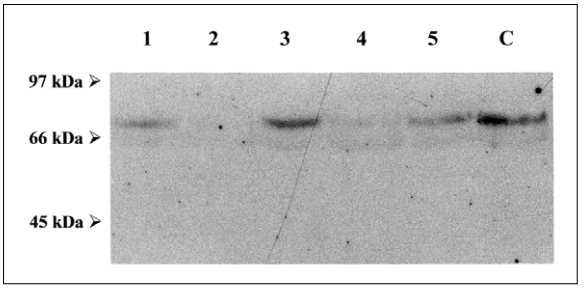


**Figure 2.** Decrease of Hsp70 in whole human saliva during stimulation. 20 μl of first control (lane 1), first stimulated (lane 2), second control (lane 3), second stimulated (lane 4), and third control (lane 5) saliva from the participants was analyzed by SDS-PAGE and Western blot analysis as described in Materials and Methods. C: positive control from hypoxic liver. Bands are representatives from 3 experiments.

## RESULTS

No differences related to gender were detected in this study. The anti Hsp70 antibody recognized a single band around 70 kDa in the saliva of all participants, and showed a single band with the same molecular weight in the liver homogenate used as positive control (Figure 1).

Stimulation significantly increased the secretory rate of saliva, compared to the baseline (resting) rate. There was a statistically non-significant decrease in the amount of total protein. There was a significant decrease of Hsp70 concentration in the saliva during stimulation (Figure 2), whereas the activity of salivary amylase increased significantly compared to baseline. Stimulation significantly increased the total protein, amylase and Hsp70 output, compared to baseline, as well as the amylase/protein ratio. The Hsp70/amylase ratio and Hsp70/protein ratio decreased significantly during both stimulation phases (Table 1).

## DISCUSSION

Our data strongly suggest that Hsp70 is present in human whole saliva. This finding is important, since knowing the extracellular effects of chaperones, such as cell protection [5,6], their impact on immune-recognition [2], and their 'repair' character [1], the cytoprotective effect of salivary Hsp70 on salivary gland cells, and on mucosal cells of the oral cavity and upper gastrointestinal tract is strongly suggested. The intact, healthy oral tissues of the participants, the sterile filtration of all samples, and the significant increase of the Hsp70 output during stimulation indicate that the possibility of bacterial or mucosal cell Hsp70, or direct blood contamination is a very unlikely reason for the presence of Hsp70 in human saliva. During stimulation, the secretory parameters of Hsp70 showed considerable independence from those of amylase (the main secretory protein of salivary gland acinar cells), and a moderate independence from the secretory parameters of total salivary protein (a mixture containing mainly several secretory proteins from acinar cells and proteins originating from blood sera, i.e. : salivary albumin [8,14]). These findings indicate that the presence of Hsp70 in saliva is not due to a secretory exocytotic process of the acinar cells. The transport of Hsp70 may involve passive transport from blood serum [8,14]. A small capacity active or passive transport from salivary gland cells is also possible.

## CONCLUSION

Our results indicate that Hsp70 present in the human saliva, but dissimilarly to amylase, is not transported by the exocytotic secretoric mechanisms of acinar cells. Passive transport mechanisms of Hsp70 from blood serum or from salivary gland cells may be the major routes of salivary Hsp70 secretion. A more detailed investigation of the mechanisms of Hsp70 transport to saliva, the investigation of the extracellular cytoprotective or refolding effects of Hsp70 on the mucosal cells and of their surface proteins in the oral cavity, and the demonstration of the presence or absence of other chaperones in the whole saliva will be the subjects of experiments we plan to conduct in the near future.

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**Table 1.** Effect of secretory stimulation on whole saliva samples in three repeated experiments with six individuals.

| | Cont 1. | Stim 1. | Cont 2. | Stim 2. | Cont 3. | P≤0.05 |
|---|---|---|---|---|---|---|
| Secretory | 0.47 | 3.83 | 0.67 | 3.73 | 0.70 | C1–S1 |
| rate | (0.12) | (0.99) | (0.28) | (1.39) | (0.35) | C1–S2 |
| (ml/min) | /0.03/ | /0.23/ | /0.07/ | /0.31/ | /0.08/ | C1–C3 |
| Protein | 0.48 | 0.40 | 0.45 | 0.37 | 0.49 | – |
| Conc. | (0.18) | (0.07) | (0.12) | (0.09) | (0.24) | |
| (mg/ml) | /0.04/ | /0.02/ | /0.03/ | /0.02/ | /0.06/ | |
| Protein | 0.22 | 1.51 | 0.28 | 1.40 | 0.35 | C1–S1 |
| Output | (0.07) | (0.38) | (0.11) | (0.66) | (0.17) | C1–S2 |
| (mg/min) | /0.02/ | /0.09/ | /0.03/ | /0.15/ | /0.04/ | C1–C3 |
| Amylase | 53.09 | 69.04 | 65.36 | 70.21 | 73.72 | C1–S1 |
| Activity | (20.50) | (17.20) | (20.33) | (21.27) | (17.89) | C1–S2 |
| (U/ml) | /4.83/ | /4.05/ | /4.79/ | /5.01/ | /4.22/ | C1–C3 |
| Amylase | 23.53 | 257.71 | 40.96 | 252.63 | 47.56 | C1–S1 |
| output | (5.95) | (76.97) | (17.40) | (121.73) | (18.45) | C1–S2 |
| (U/min) | /1.40/ | /18.14/ | /4.10/ | /28.69/ | /4.35/ | C1–C2 |
| | | | | | | C1–C3 |
| Amy/Prot | 112.51 | 174.55 | 144.59 | 196.28 | 175.21 | C1–S1 |
| (U/mg) | (31.45) | (48.87) | (16.69) | (69.70) | (79.21) | C1–S2 |
| | /7.41/ | /11.52/ | /3.93/ | /16.43/ | /18.67/ | C1–C2 |
| | | | | | | C1–C3 |
| Hsp70 | 78.24 | 26.93 | 65.05 | 30.64 | 60.01 | C1–S1 |
| Conc. | (10.58) | (12.73) | (20.85) | (12.67) | (15.08) | C1–S2 |
| (OD/band) | /2.49/ | /3.00/ | /4.91/ | /2.99/ | /3.55/ | |
| Hsp70 | 18.14 | 52.20 | 23.07 | 57.52 | 23.30 | C1–S1 |
| Output | (2.65) | (35.29) | (13.73) | (40.46) | (13.04) | C1–S2 |
| Odx10$^{-2}$/min | /0.62/ | /8.31/ | /3.24/ | /9.54/ | /3.07/ | |
| Hsp/Prot | 165.56 | 73.32 | 123.77 | 95.45 | 159.31 | C1–S1 |
| (OD/mg) | (30.73) | (35.55) | (44.33) | (62.75) | (72.38) | C1–S2 |
| | /7.24/ | /8.38/ | /10.45/ | /14.79/ | /17.06/ | |
| Hsp/Amy | 1.69 | 0.40 | 0.86 | 0.51 | 0.89 | C1–S1 |
| (OD/U) | (0.56) | (0.25) | (0.23) | (0.43) | (0.42) | C1–S2 |
| | /0.13/ | /0.06/ | /0.05/ | /0.10/ | /0.10/ | |

Cont 1.– resting state; Stim 1., Stim 2.– repeated 2 min. secretory stimulations;
Cont 2., Cont 3.– resting (recovery) phases between and after the stimulations, respectively;
Amy.– amylase activity; OD.– optical density; Prot.– total protein;
Standard deviation ( ) and standard error / / of the mean are in different parentheses respectively


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