# The role of the salivary proteome in salt sensitivity

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## Abstract

Understanding individual subjects' taste sensitivity and the mechanisms involved in peri-receptor events taking place in the oral cavity would open new avenues for the reformulation of food products. Salivary proteins are believed to interact with key food taste molecules like sodium chloride and, by doing so, seem to impact taste receptor activation. Therefore, the present study set a particular focus on the salivary proteome level before and upon chemosensory stimulation using a combination of liquid chromatography mass spectrometry and sensory experiments. Interestingly, dynamics upon stimulation and differential proteome pattern between sensitivity groups seemed to be two largely independent conditions. Gene ontology enrichment analysis of key proteins with regard to sodium chloride sensitivity revealed augmented endopeptidase activity for sensitive subjects. Non-sensitive subjects, in contrast, were high abundant in proteins showing endopeptidase inhibitor activity. In the context, increased sensitivity could be demonstrated to arise from enriched serine-type endopeptidase activity and an *in-vivo* generation of salt-modulating peptides. Decreased sensitivity, in contrast, could be correlated to increased abundances of lipocalin-1 and lysozyme C and furthermore, predicted at an individual subject's level.

## Introduction

Dietary salt intake is challenging. On the one hand, salt is essential for homeostatic regulation and nerve conductance [1]. On the other hand, excess sodium chloride has been correlated to cardiovascular diseases [2]. Understanding mechanisms involved in salivary peri-receptor events and sodium-induced ion channels, pharmacology would open new avenues to reformulate low-sodium food products without compromising on salt taste quality. Several mechanisms have been reported in terms of salt taste transduction involving the amiloride sensitive epithelial sodium chloride channel (ENaC). However, the response of the ENaC in its external environment also plays an important role. In doing so, the activity of the ENaC has been described to be modulated by serine proteases, e. g. kallikrein and trypsin, and thus, leading to facilitated signal transduction [3, 4]. Ion channels and taste receptors have further been considered to be activated by saliva during oral food processing. Alternatively, salivary constituents such as proteins or peptides may interact with sensory stimulants and therefore, influence the concentration which is available at a receptor stage [5, 6]. The wide-ranging influence of saliva on chemosensory perception raised the question as to whether the salivary composition and dynamic changes upon tastant stimulation affect salt taste perception and, drive salt taste sensitivity at an individual panellist's level. Therefore, the objective of the present study is to classify panellists according to sodium chloride sensitivity, collect saliva before and upon salty tastant stimulation and investigate time-dependent dynamic changes in the salivary proteome by means of tryptic in-solution digestion followed by protein quantitation using isobaric tags for relative and absolute quantitation (iTRAQ). The second part of this study

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focuses on sensitivity at individual panellist's level and consequently, a targeted quantitation of identified key proteins within each subject's saliva. To investigate as to whether a salt taste enhancing effect might be correlated to endoprotease-catalysed ion channel modulation or an *in-vivo* release of salt enhancing peptides, panellists were further challenged with a serine-type endoprotease and degradation products identified using sequential windowed acquisition of all theoretical mass spectra (SWATH-MS), followed by sensory evaluation.

## Experimental

*Study subjects and classification according to sodium chloride sensitivity*

31 panellists were screened in their full sodium chloride detection range by means of threshold determination using 3-alternative forced-choice tests (ISO 13301:2002) and individually adapted concentration ranges [7]. Thereupon, psychometric functions were calculated for each panellist using logistic regression models and a 95 % confidence interval and subjects classified accordingly.

*Collection of saliva and analyses of proteome pattern as well as degradation products*

The collection of unstimulated and stimulated saliva was conducted according to literature [7, 8]. The four most NaCl-sensitive ($S^+$) and four most NaCl-insensitive panellists ($S^-$) were challenged with aqueous salt solution and saliva before, upon and after chemosensory stimulation taken for tryptic in-solution digestion and shotgun proteomics as described in literature [7]. Unstimulated saliva was collected from 20 subjects classified according to NaCl-mediated salt taste sensitivity and saliva samples analysed using targeted proteomics [9]. A subset of four panellists was further challenged with trypsin (0.1 mg/mL in bottled water, 2 mL) and saliva samples before and upon trypsin challenge were analysed using SWATH-MS as described in literature [9].

## Results and Discussion

*Classification according to NaCl-mediated salt taste sensitivity*

31 healthy panellists performed 3-alternative forced-choice tests (3-AFC) with individually adapted concentration ranges to determine NaCl detection threshold concentrations. However, since detection thresholds may be subject to day-to-day variability, each panellist's reproducibility in identifying threshold level sodium chloride samples had to be evaluated prior to classification. To achieve this, logistic regression models were calculated on the basis of each subject's daily performance at respective NaCl sample concentrations. The resulting psychometric functions revealed that four panellists consistently detected NaCl concentrations of less than 1.2 mmol/L and consequently, were classified most sensitive ($S^+$). The four panellists consistently detecting NaCl concentrations above 8.1 mmol/L were classified most insensitive ($S^-$). The classification of 20 panellists into sensitive, medium sensitive non-sensitive subjects was carried out as described in literature [9].

*Salivary proteome patterns affecting salt taste sensitivity*

To investigate salt taste sensitivity in the context of the salivary proteome and dynamic changes upon salt taste stimulation, collected saliva samples were analysed using shotgun proteomics and results illustrated in figure 1.

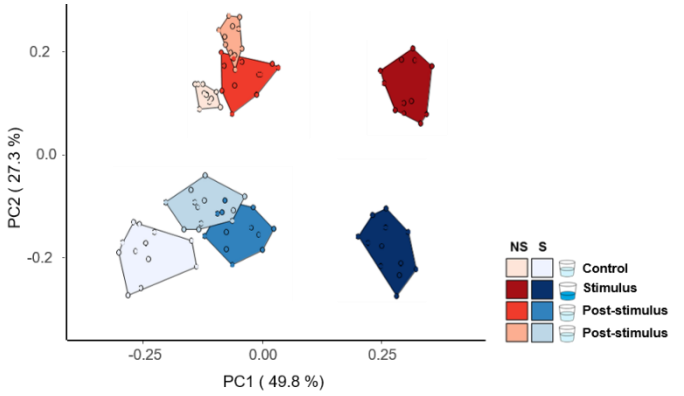


**Figure 1:** Principal component analysis of saliva samples upon salty tastant challenge

Principal component analysis demonstrated that dynamics upon stimulation (PC1) and sensitivity (PC2) seemed to be largely independent and further suggested that sodium chloride sensitivity may depend on the proteome pattern in resting saliva only. Subsequently, t-tests with a 5 % FDR cut-off revealed that lipocalin-1, lysozyme C, cystatin-D, cystatin-S and cystatin-SN are highly abundant within the $S^-$-group. Immunoglobulin heavy constant y1, cathepsin G, haptoglobin, kallikrein and myeloblastin, in contrast, were found to be key proteins for the $S^+$-group. When taken for gene ontology enrichment analysis, identified marker proteins showed significant enrichment in contrasting biological functions: The $S^+$-group demonstrated an augmented serine-type endopeptidase activity (*p-value* = $8.52 \cdot 10^{-8}$) whereas the $S^-$-group exhibited a significantly enriched cysteine-type endopeptidase inhibitor activity (*p-value* = $8.74 \cdot 10^{-9}$) and thus, suggesting that proteolytic events in the oral cavity may play a role in salt taste perception.

*Key proteins affecting individual subject's salt taste sensitivity*

To identify key proteins which may be predictive for individual panellist's sensitivity, saliva of 20 subjects classified according to NaCl-mediated salt taste was analysed by using targeted proteomics with stable isotope incorporation. In doing so, a pseudo-inverse logarithmic response between salt taste sensitivity and the abundance of lipocalin-1 and lysozyme C was found as illustrated in figure 2.

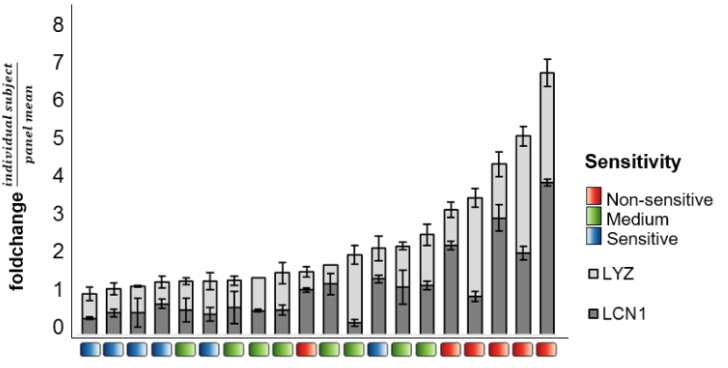


**Figure 2:** Individual panellists' sodium chloride sensitivity in correlation with abundances of lipocalin-1 and lysozyme C

Interestingly, both proteins have been demonstrated to be co-localised in salivary von Ebner glands and interact to form a thin film layer [10] which may lead to a decreased accessibility of the ENaC.

*Serine-type endopeptidases and in-vivo generation of salt taste enhancing peptides*

To answer to question as to whether enriched endopeptidase activity facilitates salt taste perception by activation of the ENaC [4] or an *in-vivo* release of salt taste enhancing peptides, a subset of four panellists was challenged with trypsin and saliva samples before and after stimulation analysed by using sequential window acquisition of all theoretical mass spectra (SWATH-MS). Interestingly, an unknown compound with a mass of 570.327 Da was observed to be significantly (*p-value* = 0.01) upregulated upon trypsin challenge demonstrating a fold change of 29. A targeted data extraction of SWATH-MS data resulted in the identification of tetrapeptide PLWR which could be confirmed by using a reference standard. A sensory evaluation of PLWR in model broth using a triangle test design [9] further revealed a salt taste enhancing effect at concentrations of 6.5 µmol/L and above. To the best of our knowledge, this is the first time that salt taste sensitivity could be correlated to an *in-vivo* endoprotease-catalysed release of salt taste modulating peptides and consequently, a facilitated salt taste perception.

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