Bioinformatics analysis and biomolecular characterization of salivary alpha amylase as risk factor for dental caries

Analisis bioinformatika dan karakterisasi biomolekuler alfa amilase saliva sebagai faktor risiko karies gigi

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ABSTRACT
Salivary alpha amylase is a component in human saliva that plays important role in carbohydrate digestion. This article aims to analyze bioinformatics and biomolecular characterization of salivary alpha amylase as risk factor of dental caries. The location of this gene is 1p21.1, with nucleotide is NC_000001.11. This information was taken from National Center for Biotechnology Information's website. The number of amino acids of salivary alpha amylase is 511, with molecular weight is 577,677.82 amu. The protein is stable with instability index is computed to be 23.58. Only 23 enzymes were predicted to be capable of cleaving the salivary alpha amylase out of a total of 37 protease. The aliphatic index is 67.12. From THMM analysis, salivary alpha amylase is found outside of membrane. It is concluded that characteristic of salivary alpha amylase can be considered as indicator for caries.
Keywords: bioinformatic, biomolecular, caries, salivary alpha amylase

ABSTRAK
Alfa amilase saliva merupakan komponen dalam saliva manusia yang berperan penting dalam pencernaan karbohidrat. Artikel ini ditujukan untuk menganalisis bioinformatika dan karakterisasi biomolekuler alfa amilase saliva sebagai faktor risiko karies gigi. Lokasi gen ini adalah 1p21.1, dengan nukleotida NC_000001.11. Informasi ini diambil dari situs web Pusat Informasi Bioteknologi Nasional. Jumlah asam amino alfa amilase saliva adalah 511, dengan berat molekul 577.677,82 kDa. Protein ini stabil dengan indeks ketidakstabilan dihitung menjadi 23,58. Hanya 23 enzim yang diprediksi mampu membelah alfa amilase saliva dari total 37 protease. Indeks alifatiknya adalah 67,12. Dari analisis THMM, alfa amilase saliva ditemukan di luar membran. Disimpulkan bahwa karakteristik alfa amilase saliva dapat dianggap sebagai indikator untuk karies.
Kata kunci: bioinformatika, biomolekuler, karies, alfa amilase saliva
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INTRODUCTION

Dental caries is the most prevalent chronic infectious disease, that occurs from adhesion of microorganism in hard tissue of oral cavity, formation of dental plaque, and the change of acidogenic environtment.[1] The lesion begins from the loss of mineral in the enamel surface and progress to become dental cavity.[2] The etiology of dental caries is multifactorial. Dental caries is closely related to the condition of oral cavity. Several host factors that can cause the development of dental caries are teeth and saliva.[1,2] Saliva contains important buffer bicarbonate that can neutralised pH in oral cavity.[3] Besides that, saliva has calcium and phospate ions those repair the loss of mineral crystals in enamel. Saliva plays critical part in maintaining dental and oral health.[3,4] Dodds *et al* stated that saliva was one of biomarker in dental and oral disease.[4]

Saliva also has many functions, such as digestive, protective, maintenance of mucous membran integrity, soft tissue repair, ecological balance, antibacterial properties, hormonal functional, excretory, and water balance because of its composition.[6] The compositions of saliva are proteins, amino acids, enzymes (such as amylase, lysozyme, glucose), immunoglobulins (such as IgA, IgG), electrolytes (such as calcium, phosphates, potassium, magnesium, bicarbonate, sodium), mucins, and nitrogenous products (such as urea, ammonia, uric acid, and creatinine).[7]

A 10-20% of the total protein content of saliva is salivary alpha amylase.[8] Salivary alpha amylase, moreover known as ptyalin, is an enzyme, produced by ephitelial acinar cells of the exocrine salivary glands.[7] It has responsibility in food digestion, through the glycogen breakdown and hydrolyses the α-glucoside bond from huge insoluble polysaccharides and oligosaccarides starch to soluble form called glucose and maltose.[9] Salivary alpha amylase is one of important part of oral fluid. Monea *et al* reported that salivary alpha amylase could be considered as indicator for dental plaque and caries experience.[8]

Salivary alpha amylase has high affinity to bind into enamel surface, and oral bacteria.[4] This condition is able to produce acidic environment due to its ability to catalyze carbohydrate in biofilm.[9] The low pH in enamel surface can cause mineral loss of hydroxyapatite. If it is not prevented, it will cause dental caries.[7,8] Thus, it is required to initiate study by examining the bioinformatics analysis and biomolecular characteristic of salivary alpha amylase.

METHODS

The genetic features of salivary alpha amylase, known as AMY1A, were obtained from the National Center for Biotechnology Information's through website www.ncbi.org. This website gave information of gene, location, nucleotide ID, and protein sequence.

Physicochemical analysis was carried out to perform analyses of the protein by using Protparam site through https://web.expasy.org/protparam/. Hydrophobicity level was analyzed by using PROTSCALE through website https://web.expasy.org/protscale/.

Analysis of transmembrane protein and target protein location were done by using membrane protein topology prediction method using THMM aplication. The link for acces the application was https://services.healthtech.dtu.dk/services/TMHMM-2.0.

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The protein structure analysis was obtained by examined the prediction of protein cleavage by protease, prediction of glycosylation index, and analyzing of the role of proteins in metabolic pathways. The prediction of protein cleavage by protease was evaluated by using PEPTIDE CUTTER, so that it could be seen kind of enzymes that were capable to cut or decompose salivary alpha amylase protein. Analysis was carried out by opening the link: https://web.expasy.org/peptide_cutter. While glycocosylation index prediction of this enzyme was assessed by opening NETNGLYC link: https://services.healthtech.dtu.dk/services/NetNGlyc-1.0gly. From this assessment, the influence protein stability and immunogenicity could be assessed. Analyze protein target locations was obtained using TARGETP by opening the link: https://services.healthtech.dtu.dk/service.php?TargetP-1.1.

Finally, the models of salivary alpha amylase was taken from https://swissmodel.expasy.org/interactive from the models, the structure of protein could also analyzed.

RESULTS

From the National Center for Biotechnology Information's through website found that salivary alpha amylase's location was 1p21.1. It meant that salivary alpha amylase was in chromosome 1 short hand, region 1, band 1, and sub-band 1. The nucleotide ID was NC_000001.11 (Fig.1).[10]

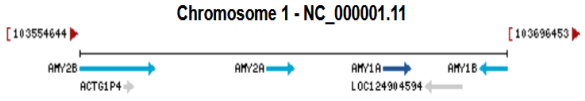

Figure 1 Location of salivary alpha amylase (AMY1A)[10]

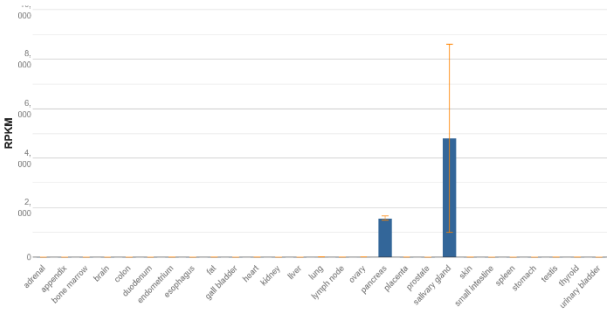

Figure 2 RPKM (Reads per kilobase of transcript per Milliom mapped reads) of salivary alpha amylase (AMY1A)[10]

The RNA-Seq of salivary alpha amylase in *Homo sapiens* was performed from 95 human individuals, which represented 27 different kind of tissue in order to evaluate tissue specificity of all protein-coding genes. It showed that salivary gland had high expression, followed by pancreas. This gene encodes salivary alpha amylase isoenzyme produced by the salivary gland (Fig.2).

The salivary alpha amylase proteins (NP_001008222.1) catalyzed 1,4-alpha-glucoside bond in insoluble polysaccaride and broken-down initial step in digestion of soluble glycogen. The amino acid sequences were shown on Fig.3.

Psychochemical characteristic of salivary alpha amylase showed that the composition of this enzyme was

Figure 3 Sequence of salivary alpha amylase[13]

seen on Table 1. The number of amino acids was 511, with molecule weight 577677.82 amu. It could be seen that total number of negatively charged residues (Asp+Glu) was 50, while total number of positively charged residues (Arg+Lys) was 5. The formula of atomic composition was $C_{2523}H_{3785}N_{701}O_{721}S_{23}$. Total number of atoms: 7753. Atomic composition of salivary alpha amylase could be seen on Table 2.

The instability index was counted to be 23.32, so that it could classify the protein as stable. The aliphatic index was 65.54 with grand average of hydropathicity (GRAVY) was -0.496. Thus, indicated that the protein was resistent as temperature rises.

The level of hydrophobicity was performed using PROTSCAL with hydropath./Kyte and Doolittle scale.[15] The hydrophobicity range of amino acids alpha amylase A1 was 10-490 (Fig.4).

Table 1 Amino acid composition of salivary alpha amylase (AMY1A) *Homo sapiens*[14]

Amino acids		Composition
Ala (A)	24	4.8%
Arg (R)	28	5.7%
Asn (N)	40	8.1%
Asp (D)	32	6.5%
Cys (C)	12	2.4%
Gln (Q)	12	2.4%
Glu (E)	18	3.6%
Gly (G)	51	10.3%
His (H)	10	2.0%
Ile (I)	25	5.1%
Leu (L)	26	5.3%
Lys (K)	31	6.3%
Met (M)	11	2.2%
Phe (F)	27	5.5%
Pro (P)	21	4.2%
Ser (S)	29	5.9%
Thr (T)	23	4.6%
Trp (W)	19	3.8%
Tyr (Y)	21	4.2%
Val (V)	35	7.1%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%

Table 2 Atomic composition of salivary alpha amylase[15]

Atom		Composition
Carbon	C	2523
Hydrogen	H	3785
Nitrogen	N	701
Oxygen	O	721
Sulfur	S	23

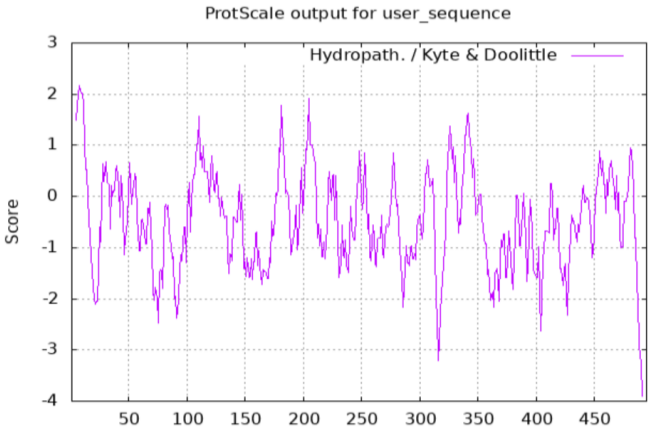

Figure 4 Proscale output of Hydrophobicity[15]

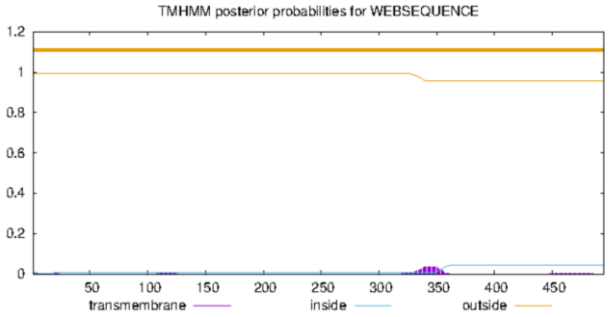

Figure 5 TMHMM web sequence of alpha amylase[16]

Transmembrane protein analysis was done using TMHMM DTU services.[16] Alpha amylase was located outside of the cell, inside of the cells, and transmembrane. Position outside of membrane were 1-495 amino acid positions, inside of the cells were 350-495 amino acid positions, and transmembrane were 325-355 amino acid position (Fig.5).

Protein structure was analyzed by peptide cutter.[17] This tool was used to evaluated kinds of enzyme that had ability to cut or elaborate alpha amylase A1 protein. Fig. 6 showed that the cleavage prediction of alpha amylase was only 23 enzymes. The enzymes were Arg-C proteinase (cleavage at site 28), Asp-N endopeptidase (cleavage at site 32), Asp-N endopeptiase + N-terminal Glu (cleavage at site 50), BNPS-Skatole (cleavage at site 19), CNBr (cleavage at site 11), Chymotrypsin-high specificity (C-term to [FYW], not before P) (cleavage at site 63), Chymotrypsin-low specificity (C-term to [FYWMML], not before P) (cleavage at site 108), Clostripain, Formic acid (cleavage at site 28), Glutamyl endopeptidase (cleavage at site 32), Hydroxylamine (cleavage at site 6), Iodosobenzoic acid (cleavage at site 19), LysC (cleavage at site 31), LysN (cleavage at site 31), NTCB (2-nitro-5thiocyanobenzoic acid) (cleavage at site 12), Pepsin (pH 1.3) (cleavage at site 80), Pepsin (pH>2) (cleavage at site 128), Proline-endopeptidase (cleavage at site 3), Proteinase K (cleavage at site 218), Staphylococcal peptidase I (cleavage at site 10), Thermolysin (cleavage at site 124), Thrombin (cleavage at site 1), and Trypsin (cleavage at site 57).

There were 14 enzymes which were not able to cleavage. The enzymes were Caspase 1, Caspase 10, Caspase 2, Caspase 3, Caspase 4, Caspase 5, Caspase 6, Caspase 7, Caspase 8, Caspase 9, Enterokinase, Factor XA, Granzyme B, and Tobacco etch virus protease. The prediction of cutting protein results by protease could be seen in Fig.6.

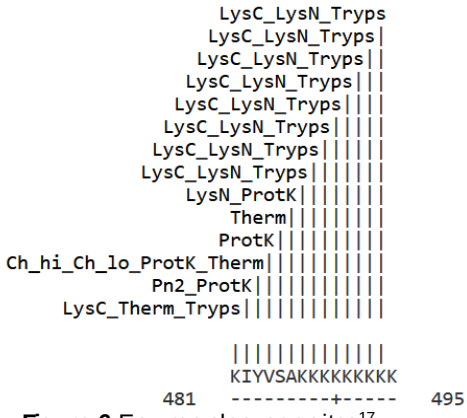

Figure 6 Enzyme cleavage sites[17]

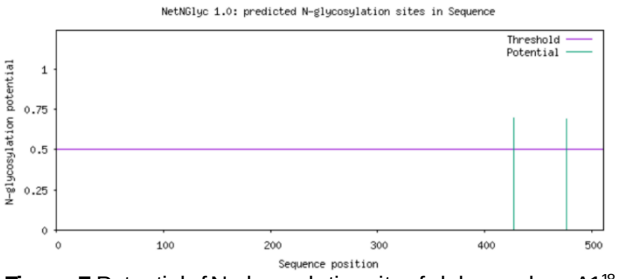

Figure 7 Potential of N-glycosylation site of alpha amylase A1[18]

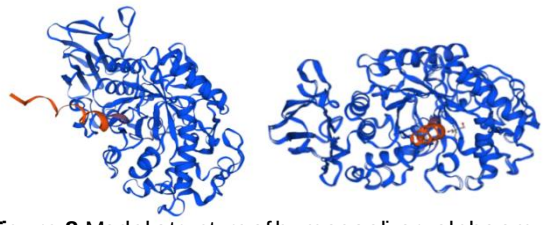

Figure 8 Model structure of human salivary alpha amylase[19]

The potential of N-glycosylation site was predicted using NETNGLYC.[18] Index of protein glycosylation was used to determine the glycosylation of protein that affected the stability and immunogenicity of protein. Fig.7 described the protein length from the N-to C-terminal was represented by the X-axis in the graph, which showed the predicted N-glycosylation sites along the protein chain. It was anticipated that a location with potential (vertical lines) that crossed the threshold (horizontal line at 0.5) was glycosylated. The alpha amylase had 2 amino acids glycosylated sites; 427 with potential gycosylated 0.69 and 476 with potential glycosylated 0.68.

The subcellular localization of eukaryotic proteins was predicted using TargetP 2.0.[19] The projected existence of any of the N-terminal presequences—secretory pathway signal peptide (SP), mitochondrial targeting peptide (mTP), or chloroplast transit peptide (cTP)—determined the location assignment. Predicted target location of alpha amylase was other location.

The structure of human salivary alpha amylase protein was analyzed. The three-dimensional atomic struc-

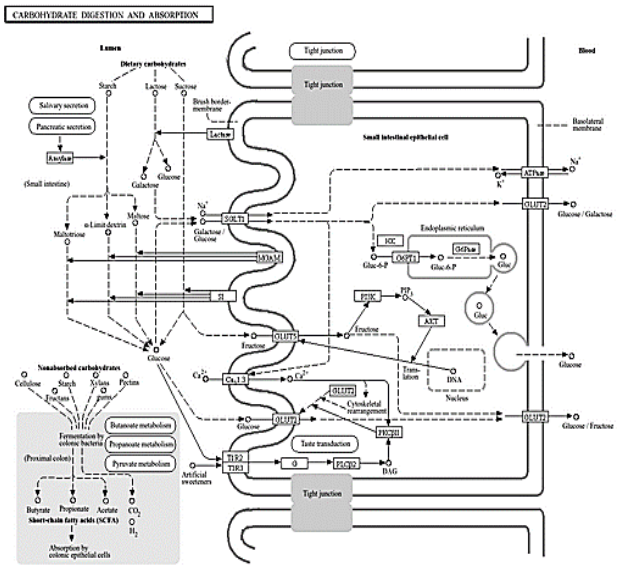

Figure 9 Human salivary alpha amylase pathway.[20]

ture of salivary amylase has been determined to understand the structure-function relationships of this enzyme. This structure was refined to an R value of 18.4% with 496 amino-acid residues, one calcium ion, one chloride ion and 170 water molecules. Investigations into the structure of human salivary alpha-amylase's Phe256Trp, consequences for the function of a conserved water molecule and its linked chain in enzyme function (Fig.8).

The role of salivary alpha amylase protein in metabolism could be found from Kyoto Encyclopedia of Genes and Genome (KEGG).[20] The KEGG, is an attempt to computerize existing understanding of biological processes and standardize gene annotations in order to link genomic information with higher level functional information. The metabolic pathway was seen on Fig.9.

DISCUSSION

One of the most abundant substances in human saliva is human salivary alpha-amylase. This enzyme has many different biological purposes.[21] The activity of human salivary alpha amylase enzymatic contributes to the carbohydrate digestion.[22] A particular class of oral *Streptococci* is bound by alpha amylase in solution with a high affinity, a property that may aid in bacterial removal and nourishment.[23] The presence of alpha-amylase in acquired enamel pellicle further implies that it plays a part in the adherence of bacteria that bind to alpha-amylase.[22,23] It appears that the intact structure of enzymes is necessary for all of these biological activities.[21]

Alpha-amylase's binding to bacteria and teeth may have significant effects on the development of dental plaque and caries.[24] The presence of alpha-amylase attached to plaque-forming bacteria may aid in the hydrolysis of dietary starch, hence supplying more glucose for the metabolism of plaque microorganisms in close proximity to the tooth surface.[23] To aid in the demineralization of teeth, the lactic acid that was so created may be added to the acidic pool found in plaque.[22]

From the website of National Center for Biotechnology Information, it could be seen that human salivary alpha amylase was mostly secreted by mayor and minor salivary gland. The characteristic of parotid saliva was high concentration of proline-rich protein (PRPs) and low levels of mucins. The α-(1,4)-glycosidic linkages in polysaccharides were hydrolyzed by alpha amylase.[25] As a result, it was crucial for initiating the process of polysaccharide's digestion in the human oral cavity, where starch was partially broken down into maltose and glucose. In this way, the enzyme could detect the sweet flavor.[26]

Beside in salivary gland, amylase could also be found in pancreas. In the human, there were five isoenzymes of amylases that assigned into family A and family B isoenzymes.[27] They had three isoforms of salivary amylase and two isoforms of pancreatic amylase. There was a discernible variation in the carbohydrate content of these families; family A had carbohydrates (62 kDa), while family B had no detectable carbohydrates (56 kDa).[26,27]

Psychochemical characteristic, amino acid composition, and atomic composition of human salivary alpha amylase showed that the protein was stabil. Protein stability was defined as the entity group of forces which preserve an equilibration to enable the protein molecule to get along in either a folded or a denatured. The stability of alpha amylase was associated with the balance of native (N) conformation and denatured (D) conformation under physiological condition such as the changes of temperature, due to its characteristic and composition.[28]

Each type of amino acid had a numerical value, which defines an amino acid scale. There were numerous more scales based on various chemical and physical properties of the amino acids, but the most commonly utilized ones were the hydrophobicity or hydrophilicity scales and the secondary structure conformational parameters scales. There were 57 preconfigured scales entered from the literature, provided by PROTSCALE.

Kyte and Doolittle stated that hydrophobicity of protein was measured as an exchange of free energy of amino acids site from apolar solvent to water and to characterize the segments of protein with nonpolar amino acids interacted with lipid bilayer.[29] The chain of amino acid apolar sites was disposed particularly into molecular interior, creating hydrophobic core, whereas the chain of amino acids polar site was arranged the outer and conform to chain turns. The hydrophobicity range of amino acids alpha amylase A1 was 10-490. It meant that the affinity of alpha amylase had higher hydrophobicity. This condition described that alpha amylase retentived and had more equilibrium constant conditon. On the other side, several studies also reported that hydrophobic chains were less resistant to adherent bacteria than hydrophilic chains.[30]

Human salivary alpha amylase location mostly in outside of membrane, because this enzyme was secreted in salivary gland, produced by acinar cells.[5] The cells were innervated by sympathetic and parasympathetic pathways.[6] Alpha amylase was increased by stimulation of sympathetic activity, while the increased of parasympathetic caused no or little effect on amylase syn-

thesis.[5,6]

The prediction potential cleavage site by peptida cutter showed that human salivary alpha amylase had 23 enzymes that were able to cleavage, whereas 14 enzymes were not able to cleavage. The peptide cutter described the position of cleavage site, peptides sequences, lenghts, and masses of the alpha amylase.[17,31]

The prediction of NETNGLYC described that the alpha amylase had 2 amino acids glycosylated sites; 427 with potential gycosylated 0.69 and 476 with potential glycosylated 0.68, which the threshold was 0.5.[18] These informations told that alpha amylase had a good physiological and pathological control, and played important role in the folding and maintaining protein. The bioactivity and its properties influenced cell adhesion, cell growth, and cell differentiation.[32]

The model of three-dimensional structure of alpha amylase showed that this protein may interact with glucosyltranferase enzymes from oral bacteria and saliva coated hydroxyapatite. This binding played important role for dental plaque amd caries formation.[33] Yazid *et al* reported that the higher human salivary alpha amylase absorption spectrum level, the higher risk factor of caries.[34] Another study also reported that alpha amylase was one of essential factor as biomarker-host-related factor for dental caries, because it had direct relationship with dental plaque and caries formation.[35] Early detection of this marker was capable to detect the initial caries, so that clinicians could prevent this small cavity to become irreversible damage.[36]

Saliva in oral cavity had so many functions in preserving the tissue integrity, maintaining from dental caries, and in digestive function.[37] Saliva was produced in salivary gland, such as parotid gland, submandibular gland, sublingual gland, and minor glands. Saliva was secreted by stimulating of neurotransmitter release from autonomic nerve endings.[6] Sympathetic stimulation produced high protein secretion in saliva including alpha amylase, due to the rising of cAMP, while parasympathetic excitation stimulated phospholipase C and led the increasing of intracelullar Ca^{2+} and caused fluid secretion containing ions and water. Parasymphatetic stimulation produced no or little amount of alpha amylase.[5,6]

Human salivary alpha amylase was closely associated with dental caries. Its phsycochemical characteristic affected biofilm in tooth surface. Its amino acids composition may lead its binding to Gram positive bacteria, especially Streptococci, in oral cavity and formed alpha amylase binding Streptococci (ABS).[37] Atomic structure of alpha amylase caused binding to tooth surface. Ahmadi-Motamayei *et al* stated that alpha amylase was found significantly higher in caries active groups as compared to caries free groups in males and females.[38] The structure showed the contribution of this enzymes in binding oral microbe to pellicle, especially Streptococci, as pioneer colonizer in dental plaque.[35] This enzyme hydrolyzed strach polysaccarides to small mollecules such as glucose. This product led acidic environment in tooth surface and formed dental caries.[36] Vacaru *et al* found that human salivary alpha amylase was one of potential indicators for caries lesion, because it had a function in the formation of dental biofilm and contributed to the maintenance of dental plaque.[39]

Interestingly, other study found the opposite concept. Culp *et al* reported that the presence of alpha amylase gave negative impact to bacteria colonization.[40] Salivary alpha amylase limited the growth of acidogenetic bacteteria, such as Streptococci in tooth surface.[41] Alpha amylase also promoted the elevation of plaque pH.[42] The interaction between enamel surface and saliva formed acquired enamel pellicle. Salivary alpha amylase localized in that pellicle binds with Streptococci expressed protein binding remains enzymatically dynamic, allowed Gram positive bacteria communicating throgh the binding protein to have access to the enzymatic products of bound amylase for catabolism, subsequently improving their competitiveness and/or giving metabolic substrates for adjacent other microbes.[41,42]

It is concluded that the bioinformatic and biomolecular characteristic and study determined that human salivary alpha amylase was considered as marker for caries risk factor.

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