

# **Korespondensi Penulis dengan Contemporary Clinical Dentistry**

## **Article History**

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|-------------------|--------------------|
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**Title** The protein level and molecular weight analysis in different children's toothpaste which probably induced hypersensitivity

**Type** Original Article

**Submission Date** 11-Nov-19 23:28

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| 3  | Peer Review        | -                      | 13/Feb/20 06:39  | 94 [94]        |
| 5  | Editorial Review   | -                      | 17/Mar/20 12:36  | 127 [33]       |
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**[ccd]:Acknowledgment of Online Submission:ccd\_394\_19**

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**Contemporary Clinical Dentistry** <editor@contempclindent.org>

Tue, Nov 12, 2019 at 6:28 AM

Reply-To: hendrik-s-b@fkg.unair.ac.id

To: hendrik-s-b@fkg.unair.ac.id

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Contemporary Clinical Dentistry has received your manuscript entitled "The children's toothpaste protein analysis using biuret test and sodium dodecyl sulfate page towards hypersensitivity incidence" for consideration for publication. The reference number for this manuscript is "ccd\_394\_19". Kindly quote this in correspondence related to this manuscript.

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Yours sincerely,  
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Fri, Apr 17, 2020 at 12:47 AM

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With reference to your manuscript entitled 'The children's toothpaste protein analysis using biuret test and sodium dodecyl sulfate page towards hypersensitivity incidence', please review the comments of the referees from our site <https://www.journalonweb.com/ccd>. The manuscript would be reconsidered after requisite modifications as per the comments and instructions provided by the journal.

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[REVIEWER]:

specific comments are given in the main article file.

in general grammatical errors need to be corrected, english language, sentence quality needs to be improved.

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Title of the article: The children's toothpaste protein analysis using biuret test and sodium dodecyl sulfate page towards hypersensitivity incidence

**Commented [SK1]:** Title needs to be more accurate well framed ... please reframe

Abstract:

Context:

A new case of allergic reaction due to the use of toothpaste was found in California, United States, causing the death of an 11-year-old girl. Ingredients contained in toothpaste suspected as a cause of allergies are milk protein compounds.

Aims:

to analyze the level and molecular weight of children's toothpaste protein and then compare with toothpaste contain recaldent and pure cow's milk regarding with the incidence of hypersensitivity.

**Commented [SK2]:** Aim of the study is not very clear.

Settings and Design:

Stratified random sampling

Methods and Material:

Samples used were children's toothpaste products in society, namely Pepsodent®, Cussons®, Enzyme®, Kodomo®, Formula®, Colgate®, the toothpaste contain recaldent of GC Tooth Moose® and pure cow's milk. Those samples were divided into 8 groups, randomly selected according to the purpose (stratified random sampling), and then coded to maintain product confidentiality. Meanwhile, samples used as comparison groups were recaldent pasta and pure cow's milk.

**Commented [SK3]:** ?

Statistical analysis used:

Nil

**Commented [SK4]:** Why the statistical analysis not considered? Test tooth pastes with comparison pastes (recaldent and pure cows milk) need to be compared for the amount of protein and the type of protein present.

Results:

Each sample was analyzed for protein content using a biuret test and protein molecular weight using the Sodium Dodecyl Sulfate (SDS-Page) test. Protein was found in samples 2, 3, 5, 6, 7 and 8 through a biuret test, with a concentration of 1.82; 1.53; 2.76; 1.92; 1.85; and 3.2 µg/mL. But, the protein bands were only found in sample 5 with a molecular weight of 20.7 kDa, sample 6 with a molecular weight of 19.1 kDa, sample 7 with a molecular weight of 17.7 kDa, and sample 8 with 2 bands namely 28.7 and 39.7 kDa.

**Commented [SK5]:** When authors say samples were analysed for protein content was it recaldent? this need to be made clear here.

Conclusions:

Toothpaste containing protein with its molecular weight similar to recaldent's are found in samples 5 and 6.

Key-words:

children's toothpaste, protein molecular weight, SDS-Page, recaldent, hypersensitivity.

Key Messages:

Allergies that occur in children are considered to be dangerous and need serious attention. A rare occurrence of allergic reaction due to the use of toothpaste causing the death of an child. Ingredients contained in toothpaste suspected as a cause of allergies are milk protein compounds used, namely recaldent.

**Commented [SK6]:** Recaldnet's molecular weight needs to be mentioned here. Pure cows milk's preotein weight is mentioned but without any context (refer introduction 3<sup>rd</sup> paragraph last sentence. )

**Commented [SK7]:** Grammar correction

Text

Introduction:

Hypersensitivity or allergy is an excessive antigenic response, which occurs in individuals who have previously experienced sensitization with certain antigens or allergens. In the last 20 years, the incidence of hypersensitivity has increased, which can cause health problems.<sup>[1,2]</sup> Allergies are caused by changes in the body's reaction (becoming vulnerable) to a material in our daily environment. Allergic contact dermatitis (ACD) due to cosmetics occurs as much as 2-4% of all dermatitis cases in the clinic, or even more.<sup>[3]</sup>

Allergens are non-parasitic foreign substances that can cause certain immune reactions in the body when they pass into the body. Conditions caused by allergens are called allergies. Allergies can cause several disorders of the mucosa, skin, digestive tract, respiratory tract, and blood vessels leading to certain symptoms, such as urticaria, dermatitis, edema, asthma, and even death.<sup>[4,5]</sup> Cosmetic contact with the skin for a long time initiates the sensitization process of some chemicals contained. Some cosmetic products that are commonly used by the public are soap, shampoo, deodorant, toothpaste, facial cream, sunscreen, and perfume.

Many cases of contact dermatitis due to allergies are caused by various cosmetic products. Irritant reactions to cosmetics usually occur in patients who have sensitive skin or a history of atopy.<sup>[6]</sup> A new and rare occurrence of allergic reaction due to the use of toothpaste was found in California, United States, causing the death of an 11-year-old girl. Ingredients contained in toothpaste suspected as a cause of allergies are milk protein compounds used, namely Recaldent or Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP).<sup>[7]</sup> These materials are used for toothpaste in patients who have teeth hypersensitivity with remineralization efficacy. Recaldent is the commercial name of CPP-ACP, a natural protein found in

**Commented [SK8]:** Was the brand of the paste used in that particular unfortunate case is known to the author? Was the comparison of all these pastes was made with that brand? Was the molecular weights compared?

**Commented [SK9]:** Are recaldent in pastes need to be compared with CPP-ACP from cow's milk as far as the molecular weight is concerned.



cow's milk.<sup>[8-10]</sup> The molecular weight of protein in cow's milk is 37 kDa is  $\alpha$ -casein, 33 kDa is  $\beta$ -casein, 46 kDa is  $\kappa$ -casein.<sup>[11]</sup>

Improving dental health at an early age is actually very important since it is one of the important elements to support holistic health, namely by maintaining dental hygiene and mouth brushing regularly at least twice a day using toothpaste. Toothpaste is generally made from a mixture of several ingredients, each of which has different functions, namely as therapy, moisturizer, water, flavor, preservative, detergent, and whitener.<sup>[12]</sup> Variations in toothpaste products sold in the market may have side effects, so parents should be able to choose the right toothpaste for children, regarding the composition of toothpaste and the age of the child.<sup>[13]</sup>

Therefore, this study aims to reveal the possibility of milk protein found in some children's toothpaste. This can be useful for the public to get as much information related to food and cosmetics that would be used, especially for children who are vulnerable to allergens, related to the development of the immune system. Toothpaste samples used were (Pepsodent®, Cussons®, Enzyme®, Kodomo®, Formula®, Colgate®).

**Commented [SK10]:** This is the main part of the study. Make this part very clear to the readers.

Subjects and Methods:

### **Toothpaste Sample Preparation**

Samples used were toothpaste products easily found in society and used by children, namely Pepsodent®, Cussons®, Enzyme®, Kodomo®, Formula®, Colgate®. Those samples then were divided into six groups randomly selected according to the objectives (stratified random sampling), and coded to maintain product confidentiality. Meanwhile, samples used as comparison groups were GC Tooth mouse® (toothpaste containing recaldent) and pure cow's milk, so that totally were eight groups.

**Commented [SK11]:** Groups should be mentioned clearly as mentioned in the table 1.

### **Analysis of Protein Levels with Biuret Test**

Measurement of protein level was carried out according to the method of Lowry et al. (1951).<sup>[14]</sup> First, 1 gram of each sample was added with 10 ml of distilled water. Second, 4 ml of each sample was added with 6 ml of biuret solution. The solution was rested for 10 minutes at 37°C, and then the protein content was analyzed by reading its absorption using a visible spectrophotometer (Thermoscientific, Germany) with a wavelength of 595 nm.

**Commented [SK12]:** Why was Biuret test chosen for protein estimation when when much accurate and less time consuming tests as Bradford test is available. Just want to know what was considered while choosing the test.

### **Protein Profile**

The protein was isolated from each sample before its protein profile was analyzed. Next, each sample was taken as much as 5 ml, and then dissolved with distilled water as much as 5 times the volume. Afterwards, the solution was sonicated with an amplitude of 20% for 10 minutes, and then centrifuged at 6000 rpm at 4°C for 15 minutes. Subsequently, supernatant was taken, and cold ethanol solution (1:1) was added. It then was stored at 4°C for 12 hours. After that, the samples were

centrifuged at a speed of 6000 rpm at 4° C for 15 minutes. The pellets then were dried until ethanol was disappeared. Finally, Tris HCl pH 6.8 (1:1) was added, and then stored at -20° C.<sup>[15]</sup>

### **Sodium Dodecyl Sulfate-Page Examination**

The protein profile was analyzed using SDS-Page with a 10% separating gel. Protein samples that had been measured for protein levels with a nanospectrophotometer (Thermoscientific, Germany) were added to the buffer sample. The samples then were heated at 70 °C for 5 minutes with a water bath. Next, electrophoresis was run at a constant current of 150 mA for 80 minutes. The distribution of the ribbon then was detected by coloring the Coomassie Brilliant Blue gel (CBBR-250).<sup>[15]</sup>

## Results:

The examination results of protein content in the toothpaste groups and the comparison groups showed that samples 2, 3, 5, 6, 7 and 8 contained protein, but there was no protein in samples 1 and 4 with biuret test (Table 1).

Electrophoresis gel shown protein bands in sample 5 with a molecular weight of 20.7 kDa, sample 6 with a molecular weight of 19.1 kDa, sample 7 with a molecular weight of 17.7 kDa, and sample 8 with 2 bands namely 28.7 and 39.7 kDa, but no protein band in sample 2 and 3 (Figure 1).

## Discussion:

Mouth and gum are parts of the digestive system contacting with food or drink first. This system has the best absorbent point of the body, thus, if there are chemicals in toothpaste, it will quickly circulate throughout the body through the bloodstream.<sup>[16,17]</sup> In some toothpaste samples, there were proteins contained with different concentrations (see Table 1). Allergy caused by a chemical material does not depend on its amount or dose. In people who have a history of allergy to a certain chemical material, making contact with a small amount of the chemical material still can cause allergic reactions.<sup>[18]</sup> Most allergens are proteins that can stimulate the body's immune response through enzymatic reactions or receptor activations on mucosal epithelial cells directly.<sup>[19]</sup>

Most food allergens contain glycoprotein. Carbohydrates covalently attach to proteins to form glycoproteins. The glycemic portion of mammalian glycoprotein is generally not immunogenic because it can be tolerated by most mammals. Mammalian immune system is able to develop tolerance to this group of carbohydrates. In contrast, non-mammalian glycoproteins, which are n@different from mammalian glycoproteins can induce IgG formation and sometimes IgE antibodies.<sup>[20]</sup>

These glycoproteins are recognized as certain antigens in the body by the immune system. The severity of allergic reactions can vary from person to person (genetic susceptibility).<sup>[21]</sup> These reactions occur after skin or mucosal contact with allergens. The allergens usually have small molecules (picric acid, dinitrochlorobenzene, different herbal ingredients, cosmetics, some drugs, metals and other substances). After being absorbed into the epidermis / epithelium, the

substance binds to protein as a carrier and becomes immunogenic, then leading to a hypersensitivity response.<sup>[22]</sup>

Based on the mechanism of immunologic reactions that occur, hypersensitivity reactions are divided into 4 groups. First, type I hypersensitivity reactions (anaphylactic reaction) participate in IgG, IgE, and Histamine. The general mechanism of this reaction is that the allergens crosslink with IgE. Mast cells and basophils then secrete vasoactive amines and other chemical mediators. Second, type II hypersensitivity reactions (cytotoxic reactions) are generally derived from the activation of the complement system after stimulation from the presence of antigen antibody complexes. IgG, IgM, and complement play a role in type 2 hypersensitivity reactions. Third, type 3 hypersensitivity reactions (immune complex reactions) occur because of the deposition of immune complexes (antigen-antibodies) that are difficult to phagocyte so that they activate complement and accumulate polymorphonuclear leukocytes in the tissues. And, type IV hypersensitivity reactions can also be called as slow cellular immune reactions since they are mediated by T CD4+ and CD8+ cells. The mechanism for this reaction in general is triggered by sensitized T lymphocytes. Cytokines and other mediators or cytotoxic then are released, mediated by direct T cells.<sup>[23-26]</sup>

The results of measurements of protein levels in groups 2, 3, 5, 6, 7 and 8 detected the presence of protein through the biuret test, but only in samples 5, 6, 7 and 8 the protein molecular weight can be determined. This can be due to low protein levels in samples 2 and 3, so the molecular weight could not be measured using the SDS-Page. The molecular weight of children's toothpaste proteins in samples 5 and 6 showed molecular weights of 20.7 and 19.1 kDa, quite close to the molecular weight of recalcant paste, 17.7 kDa. This means that the protein contained in the

toothpaste samples is likely similar to recalcified paste, so a child has a history of allergies should be warned. Meanwhile, in the cow's milk group two bands were found, 28.7 and 39.7 kDa, which showed very close to  $\alpha$ -casein and  $\beta$ -casein (see Figure 1). The addition of protein to toothpaste is intended to make toothpaste softer, and can be used as antibacterial, such as lysozyme and lactoferrin.<sup>[27]</sup>

Allergies that occur in children are considered to be dangerous and need serious attention. Based on the results of this study, there are some toothpastes containing protein with its molecular weight similar to recalcified, but the specific further investigation is required to analyze deeper whether protein used is recalcified or not.

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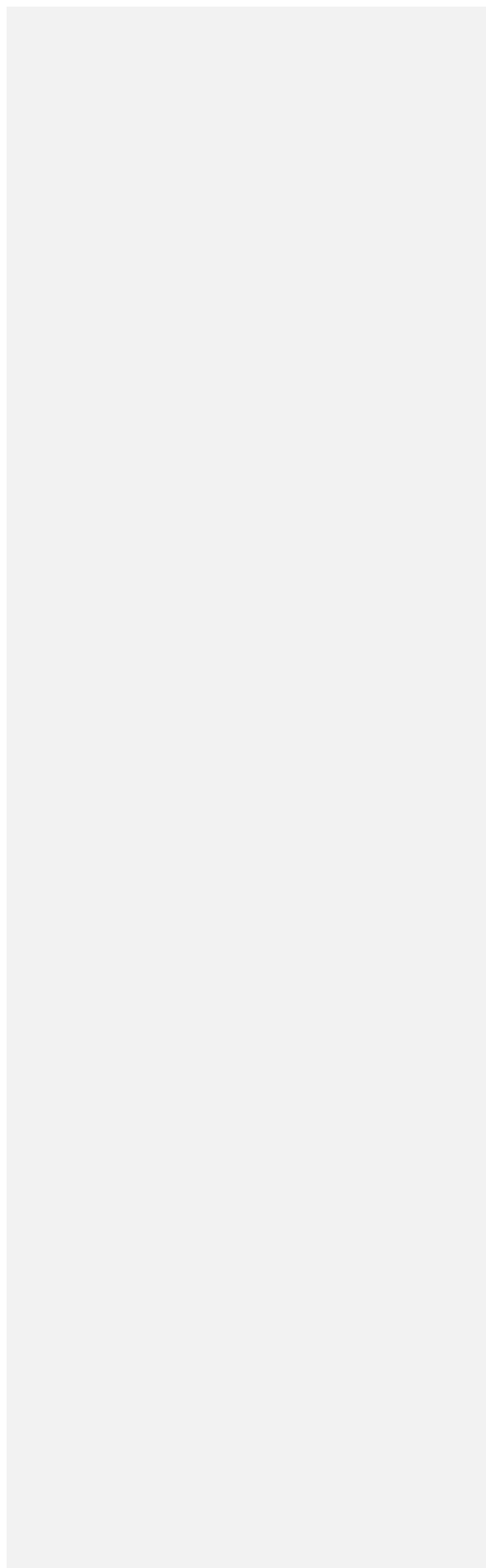


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Acknowledgement:

We thank our department colleagues for supporting this study.



Tables:

Table 1. Protein levels of children's toothpaste compared to the comparison groups

| Code     | Absorption | Level<br>( $\mu\text{g/mL}$ ) | Molecular Weight<br>(kDa) |
|----------|------------|-------------------------------|---------------------------|
| Sample 1 | -          | -                             | -                         |
| Sample 2 | 2.07       | 1.82                          | -                         |
| Sample 3 | 1.73       | 1.53                          | -                         |
| Sample 4 | -          | -                             | -                         |
| Sample 5 | 3.18       | 2.76                          | 20.7                      |
| Sample 6 | 2.19       | 1.92                          | 19.1                      |
| P1       | 2.11       | 1.85                          | 17.7                      |
| P2       | 3.71       | 3.20                          | 28.7 and 39.7             |

Note: (sign -) indicates the absence of protein through biuret test and SDS-Page.

P1 is a recaldent paste, and P2 is pure cow's milk.

Illustrations (Figures):

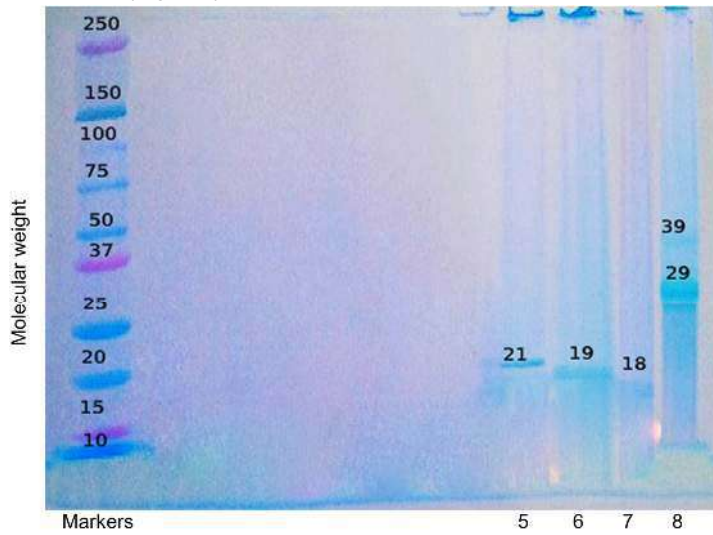


Figure 1 SDS-Page profile of protein from the children's toothpaste separated in polyacrylamide gels.

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| Reviewer Number | Original comments of the reviewer                                                                                                                                                                                                      | Reply by the author(s)                                                                                                                                                                                                       | Changes done on page number and line number |
|-----------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------|
| 1.              | Title needs to be more accurate well framed ... please reframe                                                                                                                                                                         | We thank the reviewer for the suggestion and had modified the title as follow.<br>The protein level and molecular weight analysis in different children's toothpaste which probably induced hypersensitivity                 | Page 1                                      |
| 2               | Aim of the study is not very clear. to analyze the level and molecular weight of children's toothpaste protein and then compare with toothpaste contain recaldent and pure cow's milk regarding with the incidence of hypersensitivity | We thank the reviewer for the suggestion and had revised it as follow.<br>To compare the protein level and molecular weight in children's toothpaste                                                                         | Page 1                                      |
| 3               | pasta ?                                                                                                                                                                                                                                | We thank the reviewer for the suggestion, and had revised it as follow.<br>Paste                                                                                                                                             | Page 1                                      |
| 4               | Why the statistical analysis not considered? Test tooth pastes with comparison pastes (recaldent and pure cows milk) need to be compared for the amount of protein and the type of protein present.                                    | We thank the reviewer for the suggestion we did not consider the statistical analysis because this study is to prove the presence of the protein level and compare the protein molecular weight in the children's toothpaste | Page 1                                      |
| 5               | When authors say samples were analysed for protein content was it recaldent? this need to be made clear here.                                                                                                                          | We thank the reviewer for the suggestion. We can explain that the protein content in toothpaste is compared with the similarity of the molecular protein weight in toothpaste that contains recaldent                        | Page 1                                      |
| 6               | Recaldnet's molecular weight needs to be mentioned here. Pure cows milk's preotein weight is mentioned but without any context (refer introduction 3 <sup>rd</sup> paragraph last sentence. )                                          | We thank the reviewer for the suggestion and had revised it as follow.<br>The protein molecular weight of recaldent paste is 17.7 kDa.                                                                                       | Page 2                                      |

|    |                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                                         |        |
|----|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------|
|    |                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                                                                                                                                                                                                                                                                                                                         |        |
| 7  | Grammar correction<br>An child                                                                                                                                                                                                                                                                                                                                   | We thank the reviewer for the suggestion, and had revised it as follow.<br>A child                                                                                                                                                                                                                                                                                                                                                      | Page 2 |
| 8  | Was the brand of the paste used in that particular unfortunate case is known to the author? Was the comparison of all these pastes was made with that brand? Was the molecular weights compared? death of an 11-year-old girl. Ingredients contained in toothpaste suspected as a cause of allergies are milk protein compounds used, namely recaldent or Casein | We thank the reviewer for the suggestion. We can explain that the authors use recaldent-containing paste which is well-known in Indonesia. Groups of protein-containing toothpastes shall be matched with similarities in molecular weight with the recaldent paste. Toothpaste causing death in allergic cases, because of recaldent. Recaldent is the commercial name of Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP). | Page 3 |
| 9  | Are recaldent in pastes need to be compared with CPP-ACP from cow's milk as far as the molecular weight is concerned.<br>recaldent or Casein                                                                                                                                                                                                                     | We thank the reviewer for the suggestion. We can explain that Recaldent is the commercial name of Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP).                                                                                                                                                                                                                                                                          | Page 3 |
| 10 | This is the main part of the study. Make this part very clear to the readers.<br>Recaldent is the commercial name of CPP-ACP, a natural protein found in cow's milk. The molecular weight of protein in cow's milk is 37 kDa is $\alpha$ -casein, 33 kDa is $\beta$ -casein, 46 kDa is $\kappa$ -casein                                                          | We thank the reviewer for the suggestion. We can explain that<br>The molecular weight of protein in cow's milk, 37 kDa is $\alpha$ -casein, 33 kDa is $\beta$ -casein, 46 kDa is $\kappa$ -casein, are different with Recaldent's molecular weight.                                                                                                                                                                                     | Page 3 |
| 11 | Groups should be mentioned clearly as mentioned in the table 1.<br>Those samples then were divided into six groups randomly selected                                                                                                                                                                                                                             | We thank the reviewer for the suggestion. We can explain the products confidentiality, so the groups were coded.                                                                                                                                                                                                                                                                                                                        | Page 5 |
| 12 | Why was Biuret test chosen for protein estimation when when much accurate and less time consuming                                                                                                                                                                                                                                                                | We thank the reviewer for the suggestion, that the authors use the Biuret test because                                                                                                                                                                                                                                                                                                                                                  | Page 5 |

|  |                                                                                                                                                      |                                             |  |
|--|------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------|--|
|  | tests as Bradford test is available.<br>Just want to know what was considered while choosing the test<br>Analysis of Protein Levels with Biuret Test | it is simple and already in our laboratory. |  |
|--|------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------|--|





hendrik setia budi <hendrik-s-b@fkg.unair.ac.id>

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**[ccd]:Acknowledgment for revised manuscript:ccd\_394\_19**

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# The Protein Level and Molecular Weight Analysis in Different Children's Toothpaste, which Probably Induced Hypersensitivity

## Abstract

**Context:** A new case of an allergic reaction due to the use of toothpaste was found in California, United States, causing the death of an 11-year-old girl. Ingredients contained in toothpaste suspected as a cause of allergies are milk protein compounds. **Aims:** The aim is to compare the protein level and molecular weight in children's toothpaste. **Settings and Design:** Stratified random sampling. **Subjects and Methods:** Samples used were children's toothpaste products in society, namely, Pepsodent®, Cussons®, Enzyme®, Kodomo®, Formula®, Colgate®, the toothpaste contain recaldent of GC Tooth Moose® and pure cow's milk. Those samples were divided into eight groups, randomly selected according to the purpose (stratified random sampling), and then coded to maintain product confidentiality. Meanwhile, samples used as comparison groups were recaldent paste and pure cow's milk. **Statistical Analysis Used: ???.** **Results:** Each sample was analyzed for protein content using a biuret test and protein molecular weight using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis test. The protein content in toothpaste is compared with the similarity of the molecular protein weight in toothpaste that contains recaldent. Protein was found in samples 2, 3, 5, 6, 7, and 8 through a biuret test, with a concentration of 1.82; 1.53; 2.76; 1.92; 1.85; and 3.2 µg/mL. However, the protein bands were only found in sample 5 with a molecular weight of 20.7 kDa, sample 6 with a molecular weight of 19.1 kDa, sample 7 with a molecular weight of 17.7 kDa, and sample 8 with 2 bands, namely, 28.7 and 39.7 kDa. **Conclusions:** The protein molecular weight of recaldent paste is 17.7 kDa. Toothpaste containing protein with its molecular weight similar to recaldent's are found in samples 5 and 6.

**Keywords:** Children's toothpaste, hypersensitivity, protein molecular weight, recaldent, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

## Introduction

Hypersensitivity or allergy is an excessive antigenic response, which occurs in individuals who have previously experienced sensitization with certain antigens or allergens. In the last 20 years, the incidence of hypersensitivity has increased, which can cause health problems.<sup>[1,2]</sup> Allergies are caused by changes in the body's reaction (becoming vulnerable) to material in our daily environment. Allergic contact dermatitis due to cosmetics occurs as much as 2%–4% of all dermatitis cases in the clinic, or even more.<sup>[3]</sup>

Allergens are nonparasitic foreign substances that can cause certain immune reactions in the body when they pass into the body. Conditions caused by allergens are called allergies. Allergies can cause

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several disorders of the mucosa, skin, digestive tract, respiratory tract, and blood vessels, leading to certain symptoms, such as urticaria, dermatitis, edema, asthma, and even death.<sup>[4,5]</sup> Cosmetic contact with the skin for a long time initiates the sensitization process of some chemicals contained. Some cosmetic products that are commonly used by the public are soap, shampoo, deodorant, toothpaste, facial cream, sunscreen, and perfume.

Many cases of contact dermatitis due to allergies are caused by various cosmetic products. Irritant reactions to cosmetics usually occur in patients who have sensitive skin or a history of atopy.<sup>[6]</sup> A new and rare occurrence of an allergic reaction due to the use of toothpaste was found in California, United States, causing the death of an 11-year-old girl. Ingredients contained in toothpaste suspected as a cause of allergies are milk protein compounds used, namely, recaldent

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or casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).<sup>[7]</sup> These materials are used for toothpaste in patients who have teeth hypersensitivity with remineralization efficacy. Recaldent is the commercial name of CPP-ACP, a natural protein found in cow's milk.<sup>[8-10]</sup> The molecular weight of protein in cow's milk is 37 kDa is  $\alpha$ -casein, 33 kDa is  $\beta$ -casein, 46 kDa is  $\kappa$ -casein.<sup>[11]</sup>

Improving dental health at an early age is actually very important since it is one of the important elements to support holistic health, namely by maintaining dental hygiene and mouth brushing regularly at least twice a day using toothpaste. Toothpaste is generally made from a mixture of several ingredients, each of which has different functions, namely as therapy, moisturizer, water, flavor, preservative, detergent, and whitener.<sup>[12]</sup> Variations in toothpaste products sold in the market may have side effects, so parents should be able to choose the right toothpaste for children, regarding the composition of toothpaste and the age of the child.<sup>[13]</sup>

Therefore, this study aims to reveal the possibility of milk protein found in some children's toothpaste. This can be useful for the public to get as much information related to food and cosmetics that would be used, especially for children who are vulnerable to allergens, related to the development of the immune system. Toothpaste samples used were (Pepsodent<sup>®</sup>, Cussons<sup>®</sup>, Enzyme<sup>®</sup>, Kodomo<sup>®</sup>, Formula<sup>®</sup>, Colgate<sup>®</sup>).

## Subjects and Methods

### Toothpaste sample preparation

Samples used were toothpaste products easily found in society and used by children, namely Pepsodent<sup>®</sup>, Cussons<sup>®</sup>, Enzyme<sup>®</sup>, Kodomo<sup>®</sup>, Formula<sup>®</sup>, Colgate<sup>®</sup>. Those samples were then divided into six groups randomly selected according to the objectives (stratified random sampling) and coded to maintain product confidentiality. Meanwhile, samples used as comparison groups were GC tooth mouse<sup>®</sup> (toothpaste containing recaldent) and pure cow's milk, so that totally were eight groups.

### Analysis of protein levels with biuret test

Measurement of protein level was carried out according to the method of Lowry *et al.*<sup>[14]</sup> First, 1 g of each sample was added with 10 ml of distilled water. Second, 4 ml of each sample was added with 6 ml of biuret solution. The solution was rested for 10 min at 37°C, and then, the protein content was analyzed by reading its absorption using a visible spectrophotometer (Thermoscientific, Germany) with a wavelength of 595 nm.

### Protein profile

The protein was isolated from each sample before its protein profile was analyzed. Next, each sample was taken as much as 5 ml, and then dissolved with distilled water as

much as five times the volume. Afterward, the solution was sonicated with an amplitude of 20% for 10 min, and then centrifuged at 6000 rpm at 4°C for 15 min. Subsequently, the supernatant was taken, and cold ethanol solution (1:1) was added. It was then stored at 4°C for 12 h. After that, the samples were centrifuged at a speed of 6000 rpm at 4°C for 15 min. The pellets were then dried until ethanol was disappeared. Finally, tris HCl pH 6.8 (1:1) was added, and then stored at -20°C.<sup>[15]</sup>

### Sodium dodecyl sulfate-page examination

The protein profile was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% separating gel. Protein samples that had been measured for protein levels with a nanospectrophotometer (Thermoscientific, Germany) were added to the buffer sample. The samples were then heated at 70°C for 5 min with a water bath. Next, electrophoresis was run at a constant current of 150 mA for 80 min. The distribution of the ribbon then was detected by coloring the coomassie brilliant blue gel-250.<sup>[15]</sup>

## Results

The examination results of protein content in the toothpaste groups and the comparison groups showed that samples 2, 3, 5, 6, 7, and 8 contained protein, but there was no protein in samples 1 and 4 with the biuret test [Table 1].

Electrophoresis gel shown protein bands in sample 5 with a molecular weight of 20.7 kDa, sample 6 with a molecular weight of 19.1 kDa, sample 7 with a molecular weight of 17.7 kDa, and sample 8 with 2 bands, namely. 28.7 and 39.7 kDa, but no protein band in samples 2 and 3 [Figure 1].

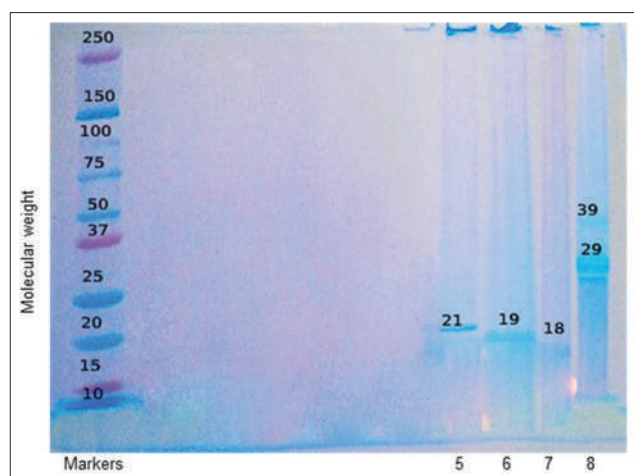
## Discussion

Mouth and gum are parts of the digestive system contacting food or drink first. This system has the best absorbent point of the body; thus, if there are chemicals in toothpaste, it will quickly circulate throughout the body through the

**Table 1: Protein levels of children's toothpaste compared to the comparison groups**

| Code     | Absorption | Level ( $\mu\text{g/mL}$ ) | Molecular weight (kDa) |
|----------|------------|----------------------------|------------------------|
| Sample 1 | -          | -                          | -                      |
| Sample 2 | 2.07       | 1.82                       | -                      |
| Sample 3 | 1.73       | 1.53                       | -                      |
| Sample 4 | -          | -                          | -                      |
| Sample 5 | 3.18       | 2.76                       | 20.7                   |
| Sample 6 | 2.19       | 1.92                       | 19.1                   |
| P1       | 2.11       | 1.85                       | 17.7                   |
| P2       | 3.71       | 3.20                       | 28.7 and 39.7          |

Sign - indicates the absence of protein through biuret test and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. P1 is a recaldent paste, and P2 is pure cow's milk. SDS: Sodium Dodecyl Sulfate



**Figure 1: Sodium dodecyl sulfate profile of protein from the children's toothpaste separated in polyacrylamide gels**

bloodstream.<sup>[16,17]</sup> In some toothpaste samples, there were proteins contained with different concentrations [Table 1]. Allergy caused by a chemical material does not depend on its amount or dose. In people who have a history of allergy to a certain chemical material, making contact with a small amount of the chemical material still can cause allergic reactions.<sup>[18]</sup> Most allergens are proteins that can stimulate the body's immune response through enzymatic reactions or receptor activations on mucosal epithelial cells directly.<sup>[19]</sup>

Most food allergens contain glycoprotein. Carbohydrates covalently attach to proteins to form glycoproteins. The glycemic portion of mammalian glycoprotein is generally not immunogenic because it can be tolerated by most mammals. The mammalian immune system can develop tolerance to this group of carbohydrates. In contrast, nonmammalian glycoproteins, which are n@different from mammalian glycoproteins, can induce immunoglobulin G (IgG) formation and sometimes IgE antibodies.<sup>[16]</sup>

These glycoproteins are recognized as certain antigens in the body by the immune system. The severity of allergic reactions can vary from person to person (genetic susceptibility).<sup>[20]</sup> These reactions occur after skin or mucosal contact with allergens. The allergens usually have small molecules (picric acid, dinitrochlorobenzene, different herbal ingredients, cosmetics, some drugs, metals, and other substances). After being absorbed into the epidermis/epithelium, the substance binds to protein as a carrier and becomes immunogenic, then leading to a hypersensitivity response.<sup>[21]</sup>

Based on the mechanism of immunologic reactions that occur, hypersensitivity reactions are divided into four Groups. First, Type I hypersensitivity reactions (anaphylactic reaction) participate in IgG, IgE, and Histamine. The general mechanism of this reaction is that the allergens crosslink with IgE. Mast cells and

basophils then secrete vasoactive amines and other chemical mediators. Second, Type II hypersensitivity reactions (cytotoxic reactions) are generally derived from the activation of the complement system after stimulation from the presence of antigen-antibody complexes. IgG, IgM, and complement play a role in Type 2 hypersensitivity reactions. Third, Type 3 hypersensitivity reactions (immune complex reactions) occur because of the deposition of immune complexes (antigen-antibodies) that are difficult to phagocyte so that they activate complement and accumulate polymorphonuclear leukocytes in the tissues. Moreover, Type IV hypersensitivity reactions can also be called as slow cellular immune reactions since they are mediated by T CD4+ and CD8+ cells. The mechanism for this reaction, in general, is triggered by sensitized T-lymphocytes. Cytokines and other mediators or cytotoxic then are released, mediated by direct T-cells.<sup>[22-25]</sup>

The results of measurements of protein levels in Groups 2, 3, 5, 6, 7, and 8 detected the presence of protein through the biuret test, but only in samples 5, 6, 7, and 8 the protein molecular weight can be determined. This can be due to low protein levels in samples 2 and 3, so the molecular weight could not be measured using the SDS-PAGE. The molecular weight of children's toothpaste proteins in samples 5 and 6 showed molecular weights of 20.7 and 19.1 kDa, quite close to the molecular weight of recalcant paste, 17.7 kDa. This means that the protein contained in the toothpaste samples is likely similar to recalcant paste, so a child has a history of allergies should be warned. Meanwhile, in the cow's milk group, two bands were found, 28.7 and 39.7 kDa, which showed very close to  $\alpha$ -casein and  $\beta$ -casein [Figure 1]. The addition of protein to toothpaste is intended to make toothpaste softer and can be used as antibacterial, such as lysozyme and lactoferrin.<sup>[26]</sup>

Allergies that occur in children are considered to be dangerous and need serious attention. Based on the results of this study, there are some toothpastes containing protein with its molecular weight similar to recalcant, but the specific further investigation is required to analyze deeper whether protein used is recalcant or not.

### Acknowledgment

We would like to thank our department colleagues for supporting this study.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflict of interest.

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| 1           | Left                  | AQ1                       | 1                                 | The children's toothpaste protein analysis using biuret test and sodium dodecyl sulfate page towards hypersensitivity incidence                                           | The Protein Level and Molecular Weight Analysis in Different Children's Toothpaste, which Probably Induced Hypersensitivity                                                                                                          |
| 1           | Right                 | AQ2                       | 11                                | Faculty of Dentistry                                                                                                                                                      |                                                                                                                                                                                                                                      |
| 1           | Right                 | AQ3                       | 42                                | Jl. Prof. Dr Moestopo 47, Surabaya, Indonesia.                                                                                                                            | Faculty of Dental Medicine, Universitas Airlangga<br>Jl. Prof. Dr Moestopo 47, Surabaya, Indonesia.                                                                                                                                  |
| 1           | Right                 | AQ4                       | 37                                |                                                                                                                                                                           | 29-May-2020                                                                                                                                                                                                                          |
| 1           | Left                  | AQ5                       | 16                                |                                                                                                                                                                           | Nil                                                                                                                                                                                                                                  |
| 1           | Left                  | AQ6                       | 22                                | The protein molecular weight of recaldent paste is 17.7 kDa. Toothpaste containing protein with its molecular weight similar to recaldent's are found in samples 5 and 6. | We found the presence of protein in children's toothpaste. The protein molecular weight of recaldent paste is 17.7 kDa. Toothpaste containing protein with its molecular weight similar to recaldent's are found in samples 5 and 6. |



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| 4 | Left  | AQ8  | 28 | Int J Mol Sci 2016;17:???                                                                                        | Int J Mol Sci 2016;17(6):915.                                                                                                                      |
| 4 | Left  | AQ9  | 32 | Sukanto. Appropriate amount and criteria of tooth paste used for early-aged children. Stomatognatic 2012;9:104-9 | Sitthisettapong T, Phantumvanit P, Huebner C, DeRouen T. Effect of CPP-ACP paste on dental caries in primary teeth. J Dent Res 2012;91(9): 847-52. |
| 4 | right | AQ10 | 37 | Sci Rep 2017;7:43344.                                                                                            | Sci Rep 2017;7:43344.<br><a href="https://doi.org/10.1038/srep43344">https://doi.org/10.1038/srep43344</a>                                         |



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# The Protein Level and Molecular Weight Analysis in Different Children's Toothpaste, which Probably Induced Hypersensitivity

## Abstract

**Context:** A new case of an allergic reaction due to the use of toothpaste was found in California, United States, causing the death of an 11-year-old girl. Ingredients contained in toothpaste suspected as a cause of allergies are milk protein compounds. **Aims:** The aim is to compare the protein level and molecular weight in children's toothpaste. **Settings and Design:** Stratified random sampling. **Subjects and Methods:** Samples used were children's toothpaste products in society, namely, Pepsodent®, Cussons®, Enzyme®, Kodomo®, Formula®, Colgate®, the toothpaste contain recalcant of GC Tooth Moose® and pure cow's milk. Those samples were divided into eight groups, randomly selected according to the purpose (stratified random sampling), and then coded to maintain product confidentiality. Meanwhile, samples used as comparison groups were recalcant paste and pure cow's milk. **Results:** Each sample was analyzed for protein content using a biuret test and protein molecular weight using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis test. The protein content in toothpaste is compared with the similarity of the molecular protein weight in toothpaste that contains recalcant. Protein was found in samples 2, 3, 5, 6, 7, and 8 through a biuret test, with a concentration of 1.82; 1.53; 2.76; 1.92; 1.85; and 3.2 µg/mL. However, the protein bands were only found in sample 5 with a molecular weight of 20.7 kDa, sample 6 with a molecular weight of 19.1 kDa, sample 7 with a molecular weight of 17.7 kDa, and sample 8 with 2 bands, namely, 28.7 and 39.7 kDa. **Conclusions:** We found the presence of protein in children's toothpaste. The protein molecular weight of recalcant paste is 17.7 kDa. Toothpaste containing protein with its molecular weight similar to recalcant's are found in samples 5 and 6.

**Keywords:** Children's toothpaste, hypersensitivity, protein molecular weight, recalcant, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

## Introduction

Hypersensitivity or allergy is an excessive antigenic response, which occurs in individuals who have previously experienced sensitization with certain antigens or allergens. In the last 20 years, the incidence of hypersensitivity has increased, which can cause health problems.<sup>[1,2]</sup> Allergies are caused by changes in the body's reaction (becoming vulnerable) to material in our daily environment. Allergic contact dermatitis due to cosmetics occurs as much as 2%–4% of all dermatitis cases in the clinic, or even more.<sup>[3]</sup>

Allergens are nonparasitic foreign substances that can cause certain immune reactions in the body when they pass into the body. Conditions caused by allergens are called allergies. Allergies can cause

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several disorders of the mucosa, skin, digestive tract, respiratory tract, and blood vessels, leading to certain symptoms, such as urticaria, dermatitis, edema, asthma, and even death.<sup>[4,5]</sup> Cosmetic contact with the skin for a long time initiates the sensitization process of some chemicals contained. Some cosmetic products that are commonly used by the public are soap, shampoo, deodorant, toothpaste, facial cream, sunscreen, and perfume.

Many cases of contact dermatitis due to allergies are caused by various cosmetic products. Irritant reactions to cosmetics usually occur in patients who have sensitive skin or a history of atopy.<sup>[6]</sup> A new and rare occurrence of an allergic reaction due to the use of toothpaste was found in California, United States, causing the death of an 11-year-old girl. Ingredients contained in toothpaste suspected as a cause of allergies are milk protein compounds used, namely, recalcant

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or casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).<sup>[7]</sup> These materials are used for toothpaste in patients who have teeth hypersensitivity with remineralization efficacy. Recaldent is the commercial name of CPP-ACP, a natural protein found in cow's milk.<sup>[8-10]</sup> The molecular weight of protein in cow's milk is 37 kDa is  $\alpha$ -casein, 33 kDa is  $\beta$ -casein, 46 kDa is  $\kappa$ -casein.<sup>[11]</sup>

Improving dental health at an early age is actually very important since it is one of the important elements to support holistic health, namely by maintaining dental hygiene and mouth brushing regularly at least twice a day using toothpaste. Toothpaste is generally made from a mixture of several ingredients, each of which has different functions, namely as therapy, moisturizer, water, flavor, preservative, detergent, and whitener.<sup>[12]</sup> Variations in toothpaste products sold in the market may have side effects, so parents should be able to choose the right toothpaste for children, regarding the composition of toothpaste and the age of the child.<sup>[13]</sup>

Therefore, this study aims to reveal the possibility of milk protein found in some children's toothpaste. This can be useful for the public to get as much information related to food and cosmetics that would be used, especially for children who are vulnerable to allergens, related to the development of the immune system. Toothpaste samples used were (Pepsodent<sup>®</sup>, Cussons<sup>®</sup>, Enzyme<sup>®</sup>, Kodomo<sup>®</sup>, Formula<sup>®</sup>, Colgate<sup>®</sup>).

## Subjects and Methods

### Toothpaste sample preparation

Samples used were toothpaste products easily found in society and used by children, namely Pepsodent<sup>®</sup>, Cussons<sup>®</sup>, Enzyme<sup>®</sup>, Kodomo<sup>®</sup>, Formula<sup>®</sup>, Colgate<sup>®</sup>. Those samples were then divided into six groups randomly selected according to the objectives (stratified random sampling) and coded to maintain product confidentiality. Meanwhile, samples used as comparison groups were GC tooth mouse<sup>®</sup> (toothpaste containing recaldent) and pure cow's milk, so that totally were eight groups.

### Analysis of protein levels with biuret test

Measurement of protein level was carried out according to the method of Lowry *et al.*<sup>[14]</sup> First, 1 g of each sample was added with 10 ml of distilled water. Second, 4 ml of each sample was added with 6 ml of biuret solution. The solution was rested for 10 min at 37°C, and then, the protein content was analyzed by reading its absorption using a visible spectrophotometer (Thermoscientific, Germany) with a wavelength of 595 nm.

### Protein profile

The protein was isolated from each sample before its protein profile was analyzed. Next, each sample was taken as much as 5 ml, and then dissolved with distilled water as

much as five times the volume. Afterward, the solution was sonicated with an amplitude of 20% for 10 min, and then centrifuged at 6000 rpm at 4°C for 15 min. Subsequently, the supernatant was taken, and cold ethanol solution (1:1) was added. It was then stored at 4°C for 12 h. After that, the samples were centrifuged at a speed of 6000 rpm at 4°C for 15 min. The pellets were then dried until ethanol was disappeared. Finally, tris HCl pH 6.8 (1:1) was added, and then stored at -20°C.<sup>[15]</sup>

### Sodium dodecyl sulfate-page examination

The protein profile was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% separating gel. Protein samples that had been measured for protein levels with a nanospectrophotometer (Thermoscientific, Germany) were added to the buffer sample. The samples were then heated at 70°C for 5 min with a water bath. Next, electrophoresis was run at a constant current of 150 mA for 80 min. The distribution of the ribbon then was detected by coloring the coomassie brilliant blue gel-250.<sup>[15]</sup>

## Results

The examination results of protein content in the toothpaste groups and the comparison groups showed that samples 2, 3, 5, 6, 7, and 8 contained protein, but there was no protein in samples 1 and 4 with the biuret test [Table 1].

Electrophoresis gel shown protein bands in sample 5 with a molecular weight of 20.7 kDa, sample 6 with a molecular weight of 19.1 kDa, sample 7 with a molecular weight of 17.7 kDa, and sample 8 with 2 bands, namely. 28.7 and 39.7 kDa, but no protein band in samples 2 and 3 [Figure 1].

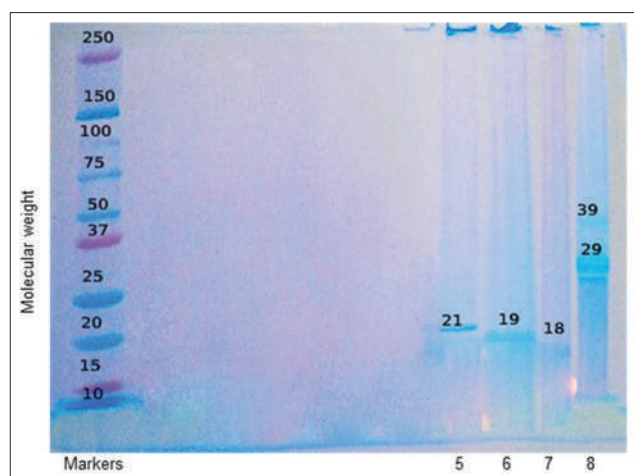
## Discussion

Mouth and gum are parts of the digestive system contacting food or drink first. This system has the best absorbent point of the body; thus, if there are chemicals in toothpaste, it will quickly circulate throughout the body through the

**Table 1: Protein levels of children's toothpaste compared to the comparison groups**

| Code     | Absorption | Level ( $\mu\text{g/mL}$ ) | Molecular weight (kDa) |
|----------|------------|----------------------------|------------------------|
| Sample 1 | -          | -                          | -                      |
| Sample 2 | 2.07       | 1.82                       | -                      |
| Sample 3 | 1.73       | 1.53                       | -                      |
| Sample 4 | -          | -                          | -                      |
| Sample 5 | 3.18       | 2.76                       | 20.7                   |
| Sample 6 | 2.19       | 1.92                       | 19.1                   |
| P1       | 2.11       | 1.85                       | 17.7                   |
| P2       | 3.71       | 3.20                       | 28.7 and 39.7          |

Sign - indicates the absence of protein through biuret test and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. P1 is a recaldent paste, and P2 is pure cow's milk. SDS: Sodium Dodecyl Sulfate



**Figure 1: Sodium dodecyl sulfate profile of protein from the children's toothpaste separated in polyacrylamide gels**

bloodstream.<sup>[16,17]</sup> In some toothpaste samples, there were proteins contained with different concentrations [Table 1]. Allergy caused by a chemical material does not depend on its amount or dose. In people who have a history of allergy to a certain chemical material, making contact with a small amount of the chemical material still can cause allergic reactions.<sup>[18]</sup> Most allergens are proteins that can stimulate the body's immune response through enzymatic reactions or receptor activations on mucosal epithelial cells directly.<sup>[19]</sup>

Most food allergens contain glycoprotein. Carbohydrates covalently attach to proteins to form glycoproteins. The glycemic portion of mammalian glycoprotein is generally not immunogenic because it can be tolerated by most mammals. The mammalian immune system can develop tolerance to this group of carbohydrates. In contrast, nonmammalian glycoproteins, which are n@different from mammalian glycoproteins, can induce immunoglobulin G (IgG) formation and sometimes IgE antibodies.<sup>[16]</sup>

These glycoproteins are recognized as certain antigens in the body by the immune system. The severity of allergic reactions can vary from person to person (genetic susceptibility).<sup>[20]</sup> These reactions occur after skin or mucosal contact with allergens. The allergens usually have small molecules (picric acid, dinitrochlorobenzene, different herbal ingredients, cosmetics, some drugs, metals, and other substances). After being absorbed into the epidermis/epithelium, the substance binds to protein as a carrier and becomes immunogenic, then leading to a hypersensitivity response.<sup>[21]</sup>

Based on the mechanism of immunologic reactions that occur, hypersensitivity reactions are divided into four Groups. First, Type I hypersensitivity reactions (anaphylactic reaction) participate in IgG, IgE, and Histamine. The general mechanism of this reaction is that the allergens crosslink with IgE. Mast cells and

basophils then secrete vasoactive amines and other chemical mediators. Second, Type II hypersensitivity reactions (cytotoxic reactions) are generally derived from the activation of the complement system after stimulation from the presence of antigen-antibody complexes. IgG, IgM, and complement play a role in Type 2 hypersensitivity reactions. Third, Type 3 hypersensitivity reactions (immune complex reactions) occur because of the deposition of immune complexes (antigen-antibodies) that are difficult to phagocyte so that they activate complement and accumulate polymorphonuclear leukocytes in the tissues. Moreover, Type IV hypersensitivity reactions can also be called as slow cellular immune reactions since they are mediated by T CD4+ and CD8+ cells. The mechanism for this reaction, in general, is triggered by sensitized T-lymphocytes. Cytokines and other mediators or cytotoxic then are released, mediated by direct T-cells.<sup>[22-25]</sup>

The results of measurements of protein levels in Groups 2, 3, 5, 6, 7, and 8 detected the presence of protein through the biuret test, but only in samples 5, 6, 7, and 8 the protein molecular weight can be determined. This can be due to low protein levels in samples 2 and 3, so the molecular weight could not be measured using the SDS-PAGE. The molecular weight of children's toothpaste proteins in samples 5 and 6 showed molecular weights of 20.7 and 19.1 kDa, quite close to the molecular weight of recalcant paste, 17.7 kDa. This means that the protein contained in the toothpaste samples is likely similar to recalcant paste, so a child has a history of allergies should be warned. Meanwhile, in the cow's milk group, two bands were found, 28.7 and 39.7 kDa, which showed very close to  $\alpha$ -casein and  $\beta$ -casein [Figure 1]. The addition of protein to toothpaste is intended to make toothpaste softer and can be used as antibacterial, such as lysozyme and lactoferrin.<sup>[26]</sup>

Allergies that occur in children are considered to be dangerous and need serious attention. Based on the results of this study, there are some toothpastes containing protein with its molecular weight similar to recalcant, but the specific further investigation is required to analyze deeper whether protein used is recalcant or not.

### Acknowledgment

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Nil.

### Conflicts of interest

There are no conflict of interest.

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## The Protein Level and Molecular Weight Analysis in Different Children's Toothpaste, which Probably Induced Hypersensitivity

### Abstract

**Context:** A new case of an allergic reaction due to the use of toothpaste was found in California, United States, causing the death of an 11-year-old girl. Ingredients contained in toothpaste suspected as a cause of allergies are milk protein compounds. **Aims:** The aim is to compare the protein level and molecular weight in children's toothpaste. **Settings and Design:** Stratified random sampling. **Subjects and Methods:** Samples used were children's toothpaste products in society, namely, Pepsodent®, Cussons®, Enzyme®, Kodomo®, Formula®, Colgate®, the toothpaste contain recaldent of GC Tooth Moose® and pure cow's milk. Those samples were divided into eight groups, randomly selected according to the purpose (stratified random sampling), and then coded to maintain product confidentiality. Meanwhile, samples used as comparison groups were recaldent paste and pure cow's milk. **Results:** Each sample was analyzed for protein content using a biuret test and protein molecular weight using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis test. The protein content in toothpaste is compared with the similarity of the molecular protein weight in toothpaste that contains recaldent. Protein was found in samples 2, 3, 5, 6, 7, and 8 through a biuret test, with a concentration of 1.82; 1.53; 2.76; 1.92; 1.85; and 3.2 µg/mL. However, the protein bands were only found in sample 5 with a molecular weight of 20.7 kDa, sample 6 with a molecular weight of 19.1 kDa, sample 7 with a molecular weight of 17.7 kDa, and sample 8 with 2 bands, namely, 28.7 and 39.7 kDa. **Conclusions:** We found the presence of protein in children's toothpaste. The protein molecular weight of recaldent paste is 17.7 kDa. Toothpaste containing protein with its molecular weight similar to recaldent's are found in samples 5 and 6.

**Keywords:** Children's toothpaste, hypersensitivity, protein molecular weight, recaldent, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

### Introduction

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or casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).<sup>[7]</sup> These materials are used for toothpaste in patients who have teeth hypersensitivity with remineralization efficacy. Recaldent is the commercial name of CPP-ACP, a natural protein found in cow's milk.<sup>[8-10]</sup> The molecular weight of protein in cow's milk is 37 kDa is  $\alpha$ -casein, 33 kDa is  $\beta$ -casein, 46 kDa is  $\kappa$ -casein.<sup>[11]</sup>

Improving dental health at an early age is actually very important since it is one of the important elements to support holistic health, namely by maintaining dental hygiene and mouth brushing regularly at least twice a day using toothpaste. Toothpaste is generally made from a mixture of several ingredients, each of which has different functions, namely as therapy, moisturizer, water, flavor, preservative, detergent, and whitener.<sup>[12]</sup> Variations in toothpaste products sold in the market may have side effects, so parents should be able to choose the right toothpaste for children, regarding the composition of toothpaste and the age of the child.<sup>[13]</sup>

Therefore, this study aims to reveal the possibility of milk protein found in some children's toothpaste. This can be useful for the public to get as much information related to food and cosmetics that would be used, especially for children who are vulnerable to allergens, related to the development of the immune system. Toothpaste samples used were (Pepsodent<sup>®</sup>, Cussons<sup>®</sup>, Enzyme<sup>®</sup>, Kodomo<sup>®</sup>, Formula<sup>®</sup>, Colgate<sup>®</sup>).

## Subjects and Methods

### Toothpaste sample preparation

Samples used were toothpaste products easily found in society and used by children, namely Pepsodent<sup>®</sup>, Cussons<sup>®</sup>, Enzyme<sup>®</sup>, Kodomo<sup>®</sup>, Formula<sup>®</sup>, Colgate<sup>®</sup>. Those samples were then divided into six groups randomly selected according to the objectives (stratified random sampling) and coded to maintain product confidentiality. Meanwhile, samples used as comparison groups were GC tooth mouse<sup>®</sup> (toothpaste containing recaldent) and pure cow's milk, so that totally were eight groups.

### Analysis of protein levels with biuret test

Measurement of protein level was carried out according to the method of Lowry *et al.*<sup>[14]</sup> First, 1 g of each sample was added with 10 ml of distilled water. Second, 4 ml of each sample was added with 6 ml of biuret solution. The solution was rested for 10 min at 37°C, and then, the protein content was analyzed by reading its absorption using a visible spectrophotometer (Thermoscientific, Germany) with a wavelength of 595 nm.

### Protein profile

The protein was isolated from each sample before its protein profile was analyzed. Next, each sample was taken as much as 5 ml, and then dissolved with distilled water as

much as five times the volume. Afterward, the solution was sonicated with an amplitude of 20% for 10 min, and then centrifuged at 6000 rpm at 4°C for 15 min. Subsequently, the supernatant was taken, and cold ethanol solution (1:1) was added. It was then stored at 4°C for 12 h. After that, the samples were centrifuged at a speed of 6000 rpm at 4°C for 15 min. The pellets were then dried until ethanol was disappeared. Finally, tris HCl pH 6.8 (1:1) was added, and then stored at -20°C.<sup>[15]</sup>

### Sodium dodecyl sulfate-page examination

The protein profile was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% separating gel. Protein samples that had been measured for protein levels with a nanospectrophotometer (Thermoscientific, Germany) were added to the buffer sample. The samples were then heated at 70°C for 5 min with a water bath. Next, electrophoresis was run at a constant current of 150 mA for 80 min. The distribution of the ribbon then was detected by coloring the coomassie brilliant blue gel-250.<sup>[15]</sup>

## Results

The examination results of protein content in the toothpaste groups and the comparison groups showed that samples 2, 3, 5, 6, 7, and 8 contained protein, but there was no protein in samples 1 and 4 with the biuret test [Table 1].

Electrophoresis gel shown protein bands in sample 5 with a molecular weight of 20.7 kDa, sample 6 with a molecular weight of 19.1 kDa, sample 7 with a molecular weight of 17.7 kDa, and sample 8 with 2 bands, namely. 28.7 and 39.7 kDa, but no protein band in samples 2 and 3 [Figure 1].

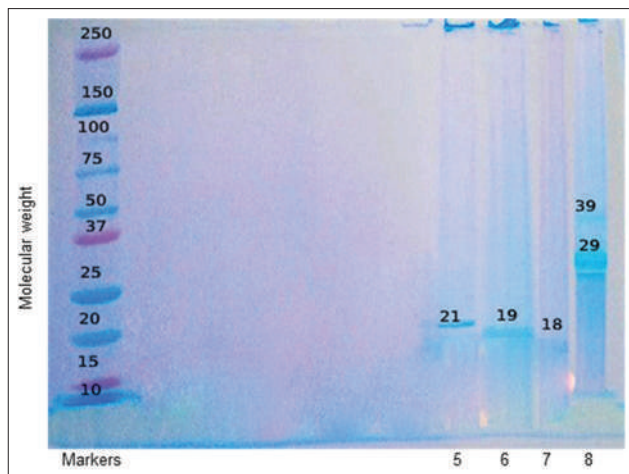
## Discussion

Mouth and gum are parts of the digestive system contacting food or drink first. This system has the best absorbent point of the body; thus, if there are chemicals in toothpaste, it will quickly circulate throughout the body through the

**Table 1: Protein levels of children's toothpaste compared to the comparison groups**

| Code     | Absorption | Level ( $\mu\text{g/mL}$ ) | Molecular weight (kDa) |
|----------|------------|----------------------------|------------------------|
| Sample 1 | -          | -                          | -                      |
| Sample 2 | 2.07       | 1.82                       | -                      |
| Sample 3 | 1.73       | 1.53                       | -                      |
| Sample 4 | -          | -                          | -                      |
| Sample 5 | 3.18       | 2.76                       | 20.7                   |
| Sample 6 | 2.19       | 1.92                       | 19.1                   |
| P1       | 2.11       | 1.85                       | 17.7                   |
| P2       | 3.71       | 3.20                       | 28.7 and 39.7          |

Sign - indicates the absence of protein through biuret test and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. P1 is a recaldent paste, and P2 is pure cow's milk. SDS: Sodium Dodecyl Sulfate



**Figure 1: Sodium dodecyl sulfate profile of protein from the children's toothpaste separated in polyacrylamide gels**

bloodstream.<sup>[16,17]</sup> In some toothpaste samples, there were proteins contained with different concentrations [Table 1]. Allergy caused by a chemical material does not depend on its amount or dose. In people who have a history of allergy to a certain chemical material, making contact with a small amount of the chemical material still can cause allergic reactions.<sup>[18]</sup> Most allergens are proteins that can stimulate the body's immune response through enzymatic reactions or receptor activations on mucosal epithelial cells directly.<sup>[19]</sup>

Most food allergens contain glycoprotein. Carbohydrates covalently attach to proteins to form glycoproteins. The glycemic portion of mammalian glycoprotein is generally not immunogenic because it can be tolerated by most mammals. The mammalian immune system can develop tolerance to this group of carbohydrates. In contrast, nonmammalian glycoproteins, which are n@different from mammalian glycoproteins, can induce immunoglobulin G (IgG) formation and sometimes IgE antibodies.<sup>[16]</sup>

These glycoproteins are recognized as certain antigens in the body by the immune system. The severity of allergic reactions can vary from person to person (genetic susceptibility).<sup>[20]</sup> These reactions occur after skin or mucosal contact with allergens. The allergens usually have small molecules (picric acid, dinitrochlorobenzene, different herbal ingredients, cosmetics, some drugs, metals, and other substances). After being absorbed into the epidermis/epithelium, the substance binds to protein as a carrier and becomes immunogenic, then leading to a hypersensitivity response.<sup>[21]</sup>

Based on the mechanism of immunologic reactions that occur, hypersensitivity reactions are divided into four Groups. First, Type I hypersensitivity reactions (anaphylactic reaction) participate in IgG, IgE, and Histamine. The general mechanism of this reaction is that the allergens crosslink with IgE. Mast cells and

basophils then secrete vasoactive amines and other chemical mediators. Second, Type II hypersensitivity reactions (cytotoxic reactions) are generally derived from the activation of the complement system after stimulation from the presence of antigen-antibody complexes. IgG, IgM, and complement play a role in Type 2 hypersensitivity reactions. Third, Type 3 hypersensitivity reactions (immune complex reactions) occur because of the deposition of immune complexes (antigen-antibodies) that are difficult to phagocyte so that they activate complement and accumulate polymorphonuclear leukocytes in the tissues. Moreover, Type IV hypersensitivity reactions can also be called as slow cellular immune reactions since they are mediated by T CD4+ and CD8+ cells. The mechanism for this reaction, in general, is triggered by sensitized T-lymphocytes. Cytokines and other mediators or cytotoxic then are released, mediated by direct T-cells.<sup>[22-25]</sup>

The results of measurements of protein levels in Groups 2, 3, 5, 6, 7, and 8 detected the presence of protein through the biuret test, but only in samples 5, 6, 7, and 8 the protein molecular weight can be determined. This can be due to low protein levels in samples 2 and 3, so the molecular weight could not be measured using the SDS-PAGE. The molecular weight of children's toothpaste proteins in samples 5 and 6 showed molecular weights of 20.7 and 19.1 kDa, quite close to the molecular weight of recalcant paste, 17.7 kDa. This means that the protein contained in the toothpaste samples is likely similar to recalcant paste, so a child has a history of allergies should be warned. Meanwhile, in the cow's milk group, two bands were found, 28.7 and 39.7 kDa, which showed very close to  $\alpha$ -casein and  $\beta$ -casein [Figure 1]. The addition of protein to toothpaste is intended to make toothpaste softer and can be used as antibacterial, such as lysozyme and lactoferrin.<sup>[26]</sup>

Allergies that occur in children are considered to be dangerous and need serious attention. Based on the results of this study, there are some toothpastes containing protein with its molecular weight similar to recalcant, but the specific further investigation is required to analyze deeper whether protein used is recalcant or not.

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Nil.

#### Conflicts of interest

There are no conflict of interest.

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