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Short communication

Hydrophobic interaction chromatography and capillary zone electrophoresis to explore the correlation between the isoenzymes of salivary α-amylase and dental caries

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Abstract

High-performance hydrophobic interaction chromatography (HIC) and capillary zone electrophoresis (CZE) were utilized in an effort to find the correlation between the composition of some isoenzymes of human salivary α-amylase (HSA) and dental caries. The mixture of more than three isoenzymes of HSA, fractionated from human parotid saliva with HIC, was further separated by CZE at the optimum pH 6.50. The composition and relative quantity of these isoenzymes were compared between two groups of individuals with different caries-susceptibility. It is found that the present frequency of peak II on CZE in the caries-free group was higher than that in the caries-active group and the relative quantities of peak III and peak IV showed remarkable differences ($p<0.05$) between the two groups. These results may indicate that the composition of HSA isoenzymes is related to the occurrence of dental caries. However, more work should be done to further affirm this correlation between the isoenzymes of salivary α-amylase and dental caries.

Keywords: Isoenzymes; α-Amylase

1. Introduction

Human salivary proteins are considerably important to dental health [1,2]. Some salivary proteins, such as proline-rich proteins, histidine-rich proteins and secretory IgA etc., have been demonstrated to be associated with dental caries [3–5], and others still need to be further studied. Human salivary α-amylase (HSA) is one of the proteins in whole saliva. It can not only catalyze starch hydrolysis, but also plays an important role in composing dental acquired enamel pellicle and binding specifically to several species of oral streptococci. These functions of HSA can lead to the formation of dental plaque and the occurrence of dental caries [6,7]. Many researchers have studied the relationship between HSA and dental caries, but no consistent conclusions have been obtained so far [8,9]. Previous studies mostly focused on the total quantity of HSA and its total enzymatic activity. Up to the present, we have not found a study on the relationship between the composition and relative quantity of HSA iso-

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enzymes and dental caries. This may be due to the poor separation among HSA isoenzymes and the lack of separation technologies with characteristics such as speed, quantification, very small sample requirement and high separation efficiency.

The studies on the constructs of the isoenzymes of human parotid α-amylase (HPA) have been reported in the literature. Kauffman et al. [10] discovered that HPA is a mixture of more than five isoenzymes, which can be separated by anionic polyacrylamide disc gel electrophoresis. Steiner and Keller [11] found, as early as 1968, that there was no individual variation in the isoenzyme patterns. Keller et al. [12] reported that HPA isoenzymes can be fractionated into two families with different molecular sizes using Bio-Gel P-100 column chromatography and polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate. The three isoenzymes which belong to family A have molecular weights of approximately 63 000. The other two isoenzymes, which belong to family B, have molecular weights of approximately 59 000. The enzymes of both families appear to be single polypeptide chains. The isoenzymes of family A contain asparagine-linked sugar chains whereas those of family B do not [12]. Yamashita et al. [13] determined the structures of single asparagine-linked sugar chains of the isoenzymes of family A by sequential exoglycosidase digestion in combination with a methylation study. Practically, the carbohydrate moieties of family A isoenzymes were released from their polypeptide portions by hydrazinolysis and labeled by reduction with $NaB[^3H]_4$, and the radioactive oligosaccharides were fractionated into one acidic and two neutral oligosaccharide fractions by paper electrophoresis and paper chromatography. Thus we may consider that HSA is made up of two major families (family A and family B). The isoenzymes of family A are glycosylated with a single *N*-linked complex carbohydrate unit, while the isoenzymes of family B appear to be nonglycosylated [10,12].

Some techniques have been employed to separate the HSA isoenzymes, including polyacrylamide gel electrophoresis (PAGE) [14], high-performance liquid chromatography (HPLC), such as size exclusion, ion-exchange [15], hydrophobic interaction chromatography [16] and isoelectric focusing electrophoresis [17]. Although fairly good separation results have been obtained, there are still some shortcomings in these techniques. Capillary electrophoresis (CE) is recognized as a new powerful analytical separation technique due to its speed, high-performance, reproducibility, automation and the requirement for a very small sample volume (typically of the order of nanoliters) [18,19]. CE has been used as an important and widely utilized separation technique for trace biological samples.

In this study, high-performance hydrophobic interaction chromatography (HIC) was used to separate HSA from human parotid saliva, and capillary zone electrophoresis (CZE) was used further to separate HSA isoenzymes from the eluate with α-amylase activity in HIC. The purpose of this study is to establish a new hyphenated method to separate the HSA isoenzymes and to attempt to explore the relationship between HSA isoenzymes and dental caries.

2. Experimental

2.1. Chemicals

Citric acid, hydrochloric acid, phosphoric acid, potassium dihydrogenphosphate, sodium orthophosphate [dimetallic], ammonium sulfate, sodium hydroxide were purchased from Xi'an Chemical Company (Xi'an, China). α-Amylase Test Reagent was from Zhongsheng Biological Technique Company (Beijing, China). Sodium dodecyl sulfate (SDS), Acrylamide, *N, N′*-methylenebisacrylamide and Coomassie Brilliant Blue G250 (Sigma, USA) were from Huamei Biological Technique Company (Xi'an, China). *N, N, N′, N*'-tetramethylethylenediamine, ammonium peroxodisulfate (APS) were from Bio-Rad Laboratories (Richmond, CA, USA). All the chemicals were analytical grade. Re-distilled water from a quartz instrument was used for all experiments. All solutions were filtered through a membrane filter (0.45 μm, Huamei Biological Technique Company, Xi'an, China) and degassed before being used in HIC and CZE.

2.2. Samples

Twenty five young Hans (the largest nationality in China) from Xi'an (China) participated in this study. They were divided into two groups, caries-free (CF)

Table 1
Distribution of the individuals in the caries-free (CF) and caries-active (CA) groups

Groups	No.	Male, *n*	Female, *n*	Age, years	DMFT[a]
CF	15	7	8	20–30	0
CA	10	5	5	20–30	6

[a] Decayed, missing and filled teeth of a individual.

group and caries-active (CA) group. Table 1 shows further details concerning the individuals.

Human parotid saliva (HPS), stimulated by regular application of a 3% w/v citric acid solution on the tongue, was collected by using a modified Lashley Cup device and centrifuged to remove insoluble materials. The supernatant was filtered through a 0.45-μm filter, then the aliquots were lyophilized, which is named as the HPS sample, and stored at −20° until needed.

2.3. High performance hydrophobic interaction chromatography (HIC)

Bio-Rad 800 HPLC apparatus (Richmond, CA, USA) was used to perform HIC separations, and a XDF-SGM hydrophobic column (4×100 mm) was from Laboratory of Modern Separation Science, Northwest University (Xi'an, China) [20]. The lyophilized HPS samples from Section 2.2 were re-dissolved in 4 *M* $(NH_4)_2SO_4$ and then were diluted with re-distilled water to a final concentration of 2 *M* $(NH_4)_2SO_4$ and 20 mg/ml lyophilized HPS, and were passed through a 0.45-μm filter before the sample was loaded. A 100-μl volume of the sample was loaded onto this column. The eluting buffers consisted of A: 2 *M* $(NH_4)_2SO_4$ in 20 m*M* potassium dihydrogenphosphate buffer, pH 7.0, followed by buffer B: 20 m*M* potassium dihydrogenphosphate buffer, pH 7.0. Elution of proteins was achieved at a flow-rate of 0.8 ml/min by a decreasing concentration of $(NH_4)_2SO_4$. A 25-min linear gradient from 100% buffer A to 100% buffer B, which was followed by a 5-min delay, was applied, with detection at 230 nm. The peak fractions were shown in Fig. 1.

We measured α-amylase activity at 37°C by using α-amylase Test Reagent box. β-Nitrophenyl-maltoheptoside was the substrate, and β-nitrophenyl was monitored by measuring the increase in absorbance at 405 nm. This catalysis reaction includes two steps. Firstly, α-amylase catalyzes the hydrolysis of β-nitrophenyl-maltoheptoside to β-nitrophenyl-maltotrioside and other compounds. Secondly, α-glucoside-enzyme catalyzes the hydrolysis of β-nitrophenyl-maltotrioside to β-nitrophenyl and other compounds [21]. According to the measurement of α-amylase activity for each peak (Fig. 1a) or the fractions that were collected in the interval of 5 min (Fig. 1b), the peak C in Fig. 1 was confirmed to contain the isoenzymes of salivary amylase, whose composition was further identified with SDS–PAGE (see Fig. 2). The fractions of the peak C in Fig. 1

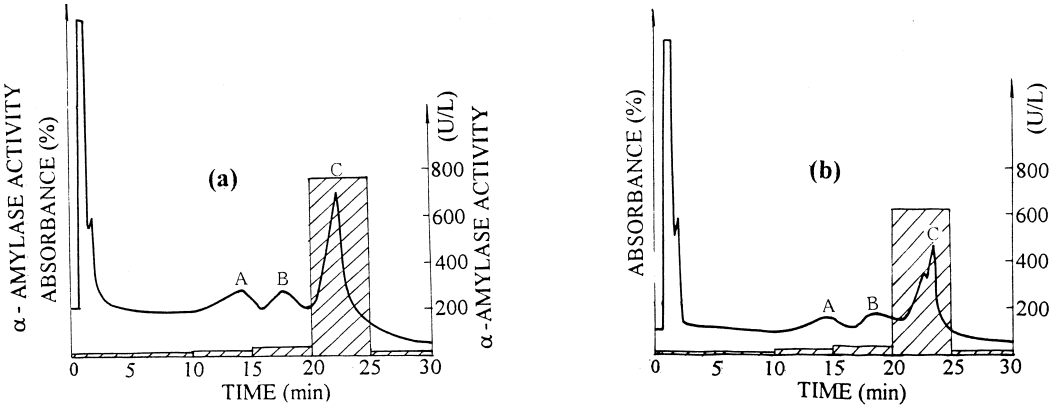

Fig. 1. Two typical profiles of protein peaks of parotid saliva from different individuals, separated by HIC on an XDF-SGM column. For sample preparation and experimental conditions see Section 2.3. *y*-axis: Absorbance (%) at 230 nm; *x*-axis: Elution time. The shaded areas represent α-amylase activity in eluates collected for each peak in Fig. 1a or in every 5 min in Fig. 1b (right-hand scale).

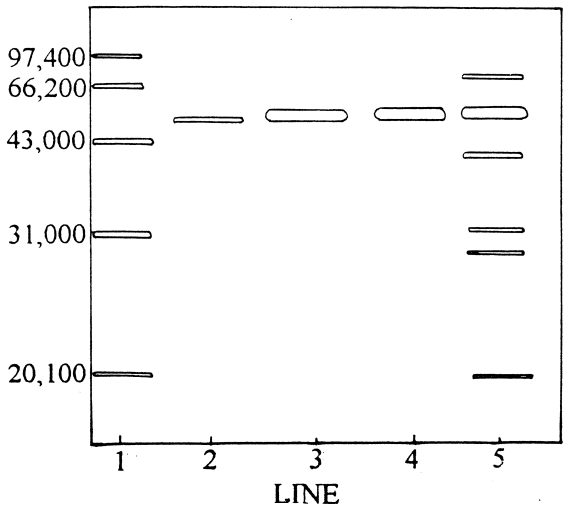

Fig. 2. SDS–PAGE of peak C obtained from HIC and the aliquots of human parotid saliva. It was performed using 15% separating gels according to the method of Laemmli [20]. Proteins in the gel were stained with Coomassie Brilliant Blue G250. Lane 1 is molecular standard (the numbers on the left-hand side are standard molecular weights); lane 2, 3 and 4 were peak C in Fig. 1 collected from different individuals on HIC, respectively; lane 5 was the aliquot of human parotid saliva for an individual of the CA group.

were prepared for SDS–PAGE by desalting with Ultra spin (molecular weight cut-off 3500, Huamei Biological Technique Company, Xi'an, China) against re-distilled water and freeze-drying, followed by dissolution in the sample media. The SDS–PAGE was done under reducing conditions according to the method of Laemmli [22] using 15% acrylamide separating gels with 10% ammonium peroxy-disulfate. The SDS–PAGE ran in the buffer, pH 8.3, containing 0.05 mol/l Tris, 0.38 mol/l glycine and 0.1% SDS. Proteins were detected by staining with Coomassie blue G-250. Then peak C in Fig. 1b was collected and dialyzed with re-distilled water to remove salt, then lyophilized again and stored at −20°, named as α-amylase isoenzymes.

2.4. Capillary zone electrophoresis (CZE)

CZE separations were performed on a NT 1229 HPCE system made in Beijing Institute of New Technology Applications, Beijing, China with a mercury-hollow cathode lamp detection at 253.7 nm, and an uncoated fused-silica capillary, 40 cm (separation length of 25 cm)×50 μm I.D., made in Yongnian, Hebei province, China. Electrolyte running solutions (pH 2.5–9.8) were prepared by using phosphate buffer (0.1 *M*). Air-cooling was used at the room temperature of 25°C, and all experiments were done at the applied voltage 12 kV.

Lyophilized samples of α-amylase isoenzymes from peak C in Fig.1 for different individuals (see Section 2.3) were respectively re-dissolved in phosphate buffer (25 m*M*) at concentration of 20 mg/ml. The injection was done for 20 s by utilizing gravity, and the height difference between liquid levels of sample and buffer vials was 0.165 m. Before using a new electrolyte running solution, the capillary was flushed with a fresh 0.1 *M* sodium hydroxide solution for 30 min and then a fresh electrolyte running solution for 2 h. After each run, the capillary was washed with 0.1 *M* sodium hydroxide for 5 min and equilibrated by flushing running electrolyte for 5 min to maintain proper reproducibility. The measurements of buffer pH were taken using a pH meter with a precision of 0.01 pH unit.

3. Results and discussion

These studies showed that the main differences among the isoenzymes of human parotid α-amylase lie mainly in their sugar chains. Thus chromatography can be used to separate these isoenzymes from other saliva proteins, but separations among these isoenzymes can be properly done by electrophoresis.

3.1. Separating the mixture of HSA isoenzymes from HPS by HIC

Proctor and Mansson's work [16], which focused on the separation of the proteins of rat parotid saliva by HIC, has shown that HIC can suitably be used in the quantification of changes in individual salivary proteins under different experimental conditions, and in the purification of individual salivary proteins. Hu at al. [23] studied the individual salivary proteins, especially HSA, and dental caries-susceptibility with HIC. One of the characteristics of HIC is that it does not lose proteins' native bioactivities nor change their conformations in separation processes. Fig. 1

shows the three chromatograms of HPS of the individuals with HIC. By measuring α-amylase activity, the α-amylase active peak of HSA was found to be mainly concentrated in peak C. Observing the HIC of different individuals, we found that peak C had slight variations. Under some favorable chromatographic conditions, peak C appeared clearly to be two peaks with relative separation (Fig. 1a). Measuring respectively α-amylase activity of the two peaks, we found that the two peaks both have higher activity of α-amylase. In some separations of individuals, peak C appeared to have double peaks with no full separation (Fig. 1b), while in others, it appeared to be a relatively wide single peak. These experimental phenomena suggested that peak C was composed of at least two components having similar hydrophobicity and activity of α-amylase. To identify the components of peak C, SDS–PAGE was done for qualitative analysis. In Fig. 2, Peak C of individuals (lane 2, 3 and 4) shows a relatively wide band, whose molecular weight ranges from 56 000 to 62 000 that agreed with the results of the literature [12,24]. Therefore, the components of peak C in HIC ought to be a group of HSA isoenzymes and could not contain other proteins. However, with the HIC system available now, the isoenzymes in peak C could not be separated fully, and SDS–PAGE of Peak C did not show a satisfactory separation. So it is necessary to employ a more efficient separation technique, whose separation mechanism must be different to HIC or SDS–PAGE.

3.2. Further separation of HSA isoenzymes with CZE

CZE was chosen to further separate HSA isoenzymes after HIC. The protein migration in CZE is based on the differences in the electric charge, molecular mass and conformation of proteins, and this migration law can be brought to light by electrophoretic mechanical migration model [25,26]. The optimization of CE systems requires complex system engineering [27], in which buffer pH is an important parameter. The buffer pH remarkably affects the separation efficiency of CZE by (1) changing the net charge of solute, and (2) changing electroosmosis and the protein-wall interaction [18,19]. Thus the manipulation of buffer pH often becomes a key strategy in CZE optimization.

Fig. 3 shows CZE electropherograms of peak C in Fig. 1 (see Section 2.4) of the same individual at several pHs from 2.5 to 9.8. The most efficient

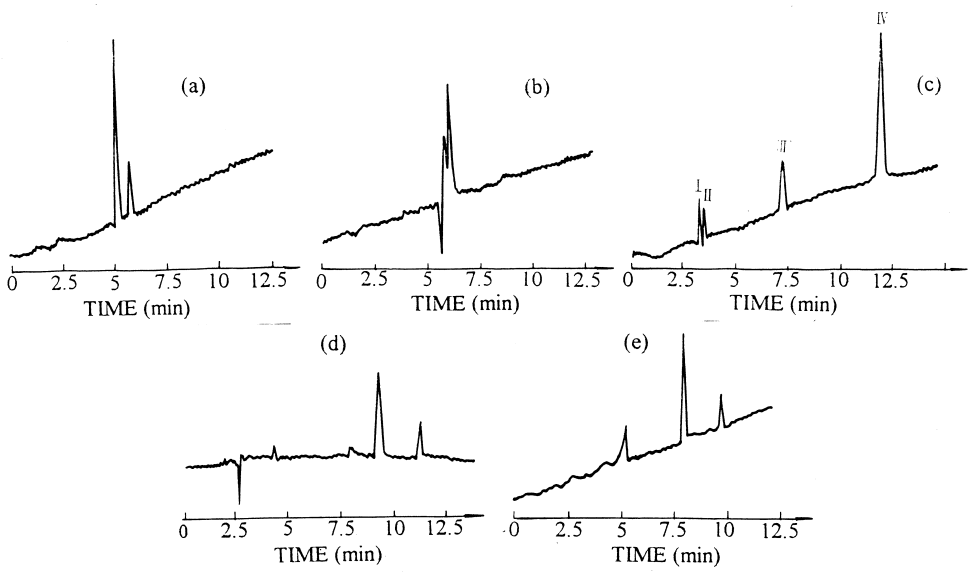

Fig. 3. Effect of buffer pH on the separation of HSA isoenzymes in CZE. For sample preparation and experimental conditions see Section 2.4. Buffer pH: (a) 2.5 ; (b) 4.5; (c) 6.5; (d) 8.5; (e) 9.8.

separation was achieved at pH 6.5, at which four peaks were separated with satisfactory resolution. In other pH conditions, the number of peaks is less than that at pH 6.5 or the resolutions are poor. The literature [10,12,14,16] shows that no distinct differences were observed in the amino acid composition among the isoenzymes, and the major difference was in asparagine-linked sugar chains. Therefore, we could speculate, for every isoenzyme, that the degree at which buffer pH correspondingly affects their conformation should be similar in this CZE. So the effect of pH on separation may be mainly due to the changing of the charges of sugar chains of HSA isoenzymes. According to previous research [17,28], the p*I* range of the isoenzymes is about 5.5 to 7.0. While the buffer pH is within the p*I* range, the isoenzymes with different p*I* may have charges of different sign of, which can result in obvious differences in migration. Therefore better separation can be gained at pH 6.5.

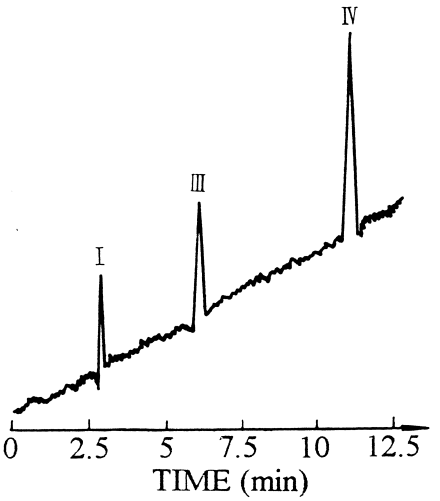

Fig. 4. Electropherograms of HSA isoenzymes from an individual obtained with 0.1 *M* phosphate buffer at pH 6.50. Other operating conditions were as described in Section 2.4.

3.3. Applications and limitations of the separation of HSA isoenzymes by CZE after HIC

This method was mainly developed to find correlation between HSA isoenzymes and dental caries. Purification of the samples was performed as described in the Section 2. Measuring α-amylase activity and the validation of SDS–PAGE (see Section 2.3) were routine methods to identify HSA isoenzymes on HIC [16] and Sephadex G-75 gel permeation chromatography [13]. Thus we are tempted to affirm that Peak C of HIC in Fig. 1 is only composed of HSA isoenzymes from measurements of α-amylase activity and the validation of SDS–PAGE. Of course, after removing salt and lyophilizing, the samples of HSA isoenzymes from HIC are still a mixture of HSA isoenzymes. An inevitable deduction is that all peaks in CZE with detection wavelength 253.7 nm are the isoenzymes of HSA. The premise that the deduction is based on is that HSA isoenzymes do not change their conformations in separation processes. In routine CZE experiments, no intermediate transition of an isoenzyme could appear between the fully folded and fully unfolded states, when the CZE buffer contains no protein denaturing reagents, such as urea and GuaHCl [29–31]. Thus one peak in Figs. 3 and 4 should represent one isoenzyme in our study.

Some individuals have four peaks (refer to Fig. 3c) and others have three, peak II is not present in Fig.4. Peak II was found to be present at a frequency of approximately 53% in the CF group and at a frequency of 10% in the CA group. A significant difference ($P<0.05$) was gained by using exact probabilities in 2×2 table. This experimental result suggests that the composition of HSA isoenzymes may be related to dental caries. The other three peaks can be found in all individuals.

We chose one peak as internal standard to calculate the relative quantity of the other peaks in Figs. 3 and 4, and the statistical data were presented in Table 2. In the *t*-test, the bound value of *t* is 2.069 at the significance level $P\sim 0.05$ (both sides) when the total samples are 25. In Table 2, the *t* value of the peak height ratio for peak IV to III is 2.551, that for peak III to IV is 2.342. Both the *t* values here are bigger than the bound value of *t* (2.069). Therefore significant differences were found between the CF group and the CA group for a pair of peaks III and IV. The relative quantity of peak III in the CA group is higher than that in the CF group and the relative quantity of peak IV in the CA group is lower than that in the CF group. This result indicates that the

Table 2
Statistical data of the ratio of the heights of two peaks in CZE for different individuals

	I[a]				III				IV			
	h_{III}/h_I[b]		h_{IV}/h_I		h_I/h_{III}		h_{IV}/h_{III}		h_I/h_{IV}		h_{III}/h_{IV}	
	CF	CA	CF	CA	CF	CA	CF	CA	CF	CA	CF	CA
Mean	0.659	0.648	1.557	1.257	1.920	1.804	2.369	1.919	0.861	0.957	0.442	0.531
SD	0.356	0.311	0.801	0.681	0.950	0.652	0.515	0.250	0.495	0.349	0.100	0.082
t[c]	0.080		0.974		0.335		2.551		0.530		2.342	

[a] The number of the peak in Fig. 4, chosen as internal standard.
[b] The ratio of the heights of two peaks, the subscript of h is the number of the peak in Fig. 4.
[c] The statistical method adopted here was the t-test of mean comparison between the two groups.

proportion of HSA isoenzymes may be related to dental caries.

However, there are obvious limitations in this research. First, there is a problem in measuring the activity of each isoenzyme after CZE separation, so it is difficult to directly affirm HSA isoenzymes with CZE if the standard samples of HSA isoenzymes are absent. The combination of capillary electrophoresis and mass spectrometry (CE–MS) should be a powerful tool in further study. The specific antibody assay would be used in further studies to discover the relationships between the isoenzymes and dental caries. Second, more individual samples of CA and CF groups should be used to further affirm the correlation between the HSA isoenzymes and dental caries. Thus this study indicates that more work should be done to investigate the composition and proportion of HSA isoenzymes and dental caries.

4. Conclusion

The combination of HIC and CZE was adopted to study the relationship between HSA isoenzymes and caries susceptibility. The HSA isoenzymes were separated from HPS by HIC, and they were further separated by CZE by using phosphate buffer (0.1 *M*, pH 6.50). The results primarily showed that the composition and relative quantity of HSA isoenzymes are significantly different between CF and CA groups, which may indicate that the composition and proportion of HSA isoenzymes is closely related to dental caries. More work needs to be done in further research on this topic, such as directly affirming HSA isoenzymes by CE–MS and carrying out the research with more individual samples.

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