Differences between Cystic Fibrosis and Normal Saliva α -Amylase as a Function of Age and Sex

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Summary

α -Amylase activity and protein concentration of cystic fibrosis (CF), obligate heterozygote (H), and normal (N) saliva have been evaluated with respect to genotype, age, sex and clinical status. "Factors" present in CF saliva had no detectable effect on salivary amylase activity under reaction conditions optimized for both competitive and noncompetitive inhibition. Tris [tris-(hydroxymethyl)aminomethane] inhibited amylase activity with a $K_i = 59 \pm 10$ mM, but CF "factors" had no detectable effect on either substrate or Tris binding to α -amylase.

α -Amylase concentration and protein content of CF saliva had bimodal distributions. Thirty-six percent of the CF patients were found to be amylase hypersecretors, with amylase concentration 3 or more SD above the N mean. Amylase hypersecretion was marginally correlated with both genetic factors (with parent values, $P = 0.075$) and clinical status (with Shwachman scores, $P = 0.068$). All the hypersecretors were in the 14-24 year age group. Amylase concentration for this age was significantly higher in CF samples (mean $\pm$ SD), 82 ± 37 starch units/ml ($n = 12$), than in N samples, 33 ± 17 starch units/ml ($n = 13$), as measured by the Student t -test ($P < 0.001$). Salivary protein content of 14- to 24-year-old CF males was elevated compared to all other groups [1.57 ± 0.76 ($n = 5$) vs. 0.63 ± 0.47 ($n = 20$); Student t -test, $P < 0.005$]. Mean specific activity of male saliva dropped sharply as age increased from 10-15 years, from over 200 to less than 75 starch units/mg protein. Mean normal female specific activity values were essentially constant [97 ± 50 ($n = 18$) starch units/mg protein] from 5-30 years of age. CF male specific activity values decreased similarly to the N male values from preadolescence to adolescence, but the mean CF female values peaked later, at 17 years, with a maximum of 171 ± 58 ($n = 4$). This is significantly higher than all the other groups in the 16- to 20-year age range: 63 ± 30 ($n = 9$), $P < 0.005$. These data emphasize the need for age- and sex-matched controls in all studies of salivary composition.

Speculation

We were unable to detect any effect of a salivary CF factor on the enzymatic activity of α -amylase. Hypersecretion of salivary α -amylase was observed in 36% of CF patients. This may reflect alterations in secretory function basic to CF, or be secondary to the disease process and result from decreased secretion from the mucous salivary glands or deterioration of pancreatic function.

Cystic fibrosis (CF) is one of the most common lethal genetic diseases of man. The basic defect is not yet known. It is characterized by aberrant exocrine gland activity, progressive pulmonary disability, and gastrointestinal malfunction (5, 31). One manifestation of the abnormal exocrine gland activity is the high electrolyte concentration of CF sweat. The sweat salt concentration is the prime diagnostic indicator of CF (14). Pancreatic exocrine function is altered in most but not all CF patients (5, 31). Salivary

exocrine secretions have also been found to have altered electrolyte and protein content (31). α -Amylase (1,4- α -D-glucan 4-glucanohydrolase, EC 3.2.1.1) has been reported to be elevated in CF saliva (2, 18). Whether the elevated α -amylase in CF is a reflection of a general increase in secretory protein concentration or a more specific manifestation of CF remains to be determined.

Ciliary dyskinesia factor(s) (CDF) have been found in the serum, saliva, and urine of CF homozygotes and heterozygotes. These CF body fluids interfere with the normal beat of various ciliated preparations (9, 19, 28). The salivary CDF appears to be associated with α -amylase (1, 8, 9, 11). α -Amylase purified from CF but not N saliva possesses CDF activity on oyster gills (1, 21). CDF and α -amylase activities coprecipitate from saliva (8, 11). The two activities can be separated by extended dialysis (11). The small molecular weight salivary CDF fraction is capable of inhibiting mammalian debranching enzyme (11, 32).

Because of the close association of CDF and α -amylase in CF saliva, it was of interest to determine whether the presence of CDF could be detected in whole saliva by its effect on α -amylase activity. If CDF were found to affect α -amylase, this effect could be used to quantitate its concentration in whole saliva. The CDF effect on debranching enzyme indicates that the factor is capable of inhibiting carbohydrase activity (11). The standard saccharogenic assay for α -amylase is not affected by CDF in the saliva sample (11). A new assay for α -amylase has been developed which should be optimal for detecting CDF if this factor acts as a competitive or noncompetitive inhibitor of α -amylase (12). This assay has been used to determine the effect of CDF on salivary α -amylase activity. In conjunction with this study, the saccharogenic α -amylase activity and the protein concentration of CF, H, and N saliva samples have also been determined, and analyzed as a function of genotype, age, sex, and clinical status of the saliva donor. The results presented here indicate that the magnitude of the differences between CF and N salivary α -amylase and protein are a function of age and sex.

MATERIALS AND METHODS

CF saliva samples were donated by patients treated at the Cystic Fibrosis Center, University of California at Los Angeles (UCLA) School of Medicine. Obligate heterozygote (H) saliva samples were obtained from parents of CF patients. Normal (N) saliva samples were obtained from donors with no family history of CF. The Human Subject Protection Committee at UCLA has approved the protocol and the Informed Consent Form used for saliva collection.

α -Amylase starch activity was determined at 30° in 50 mM maleate buffer, pH 6.5, using a modification of the Fisher and Stein saccharogenic procedure (10, 12). In this reaction mixture, a 1:4000 dilution of saliva is usually used. The starch substrate is at saturating concentration with respect to the reaction rate. The resulting increase in reducing sugar concentration is determined using a modification of the Nelson-Somogyi procedure, with

glucose as standard (23). Amylase concentration in starch units per liter is defined as milligrams of glucose reducing equivalents produced per min per liter of saliva (12).

α -Amylase *p*-nitrophenyl α -maltoide (PNPM) activity was determined using a new direct spectrophotometric method (12). Reaction conditions for this assay were optimized for possible CF factor inhibition of α -amylase. The 1-ml reaction mixture contained 0.5 ml whole saliva supernate. The reaction rate is first order with respect to PNPM substrate concentration. α -Amylase activity in PNPM units per liter is defined as micromoles of *p*-nitrophenol produced per min per liter of saliva at a substrate concentration of 1.0 mM (12).

The protein content of saliva (milligrams of protein per ml saliva) was determined using Miller's (20) modification of the Lowry procedure (16). Amylase specific activity is defined as starch units per mg protein.

The following materials were purchased from the noted suppliers: PNPM, Regis Chemical Company, Morton Grove, IL; *p*-nitrophenol, Calbiochem, A Grade; starch, Merck soluble starch; gelatin, J. T. Baker, USP grade. All other reagents were of commercially available reagent grade or better. Absorbance was measured with a Gilford 240 recording spectrophotometer, equipped with a thermostatted cell compartment, automatic cell changer, and a digital readout. pH values were measured at ambient room temperature, 22–24°, with a Radiometer PHM62 standard pH meter. Distilled deionized water was used to prepare all solutions.

Multiple regression analysis was done using the BMDPIR program (7) at the Health Sciences Computing Facility, UCLA.

RESULTS

INHIBITION OF CF α -AMYLASE

Salivary α -amylase action on starch and PNPM has been compared to determine whether CDF in saliva could be detected by alterations in α -amylase catalytic activity. To determine whether CDF inhibits α -amylase in saliva, a recently developed assay (12) which should be most sensitive to inhibition was used. For simple enzyme kinetics, one of three types of inhibition patterns are usually observed: noncompetitive, competitive, and uncompetitive (15). These three modes of inhibition have different sensitivities to the inhibitor at different substrate concentrations. The patterns are summarized for saturating and first order substrate concentrations in Table 1.

The starch assay is done under saturating substrate concentration and a 1:4000 dilution of saliva in the reaction mixture. At this dilution the CDF does not affect the amylase starch activity (11). Therefore, the starch activity serves as a measure of the total uninhibited salivary amylase activity.

The PNPM activity is determined under first order substrate conditions, with a 1:1 dilution of saliva (12). These conditions of

Table 1. Effect of inhibitors at saturating and first order substrate concentrations¹

Type of inhibition	Limit for saturating substrate $S \gg K_m$		Limit for first order in substrate $K_m \gg S$	
	V_i/V_o	Observed inhibition	V_i/V_o	Observed inhibition
Noncompetitive	$\frac{1}{1 + i/K_i}$	Maximum	$\frac{1}{1 + i/K_i}$	Maximum
Competitive	1	None	$\frac{1}{1 + i/K_i}$	Maximum
Uncompetitive	$\frac{1}{1 + i/K_i}$	Maximum	1	None

¹ S: substrate concentration; K_m : Michaelis constant (15); K_i : dissociation constant of the inhibitor from the enzyme; V_i/V_o : ratio of reaction rates in the presence and absence of inhibitor; i : inhibitor concentration.

Table 2. Effect of CDF and Tris on α -amylase PNPM activity

Saliva sample genotype	CDF effect, PNPM units/starch units		Tris effect, % inhibition by 90 mM Tris	
	<i>n</i>	(mean $\pm$ SD) $\times 10^{-5}$	<i>n</i>	Mean $\pm$ SD
CF patients (CF)	24	8.8 ± 1.4	6	66 ± 4
CF parents (H)	21	9.3 ± 1.4	6	62 ± 10
Normal controls (N)	28	9.2 ± 1.5	6	64 ± 11

low substrate and high CDF concentration should be close to the optimum for detection of either noncompetitive or competitive CDF inhibition (Table 1). Uncompetitive inhibition will not be detected by comparison of the starch and PNPM activities. This mode of inhibition would be detected by comparing the PNPM activities under first order and non-first order substrate conditions.

The effect of Tris on the α -amylase activity as measured by the starch and PNPM assays confirms that amylase does differ in its sensitivity to inhibitors under the two sets of reaction conditions. Tris (90 mM) inhibits starch hydrolysis minimally ($11 \pm 10\%$), but markedly inhibits PNPM hydrolysis ($64 \pm 11\%$). This indicates that Tris is a competitive inhibitor of α -amylase. A Dixon plot (6) at six Tris concentrations from 0–180 mM yields a K_i of 59 ± 10 mM for Tris inhibition of amylase PNPM activity.

The ratio of PNPM activity to starch activity for CF, H, and N saliva would indicate whether the CDF in CF and H saliva acts as a competitive or noncompetitive inhibitor of the amylase PNPM activity (Table 2). The CF and N values are not significantly different. Thus there is neither competitive nor noncompetitive inhibition of PNPM α -amylase activity by CDF. This was examined more vigorously by analysis of variance of the regression coefficients of PNPM units vs. starch units over the three groups. Neither the slopes nor the intercepts differed among the groups ($P = 0.920$). We must conclude that even under reaction conditions which should be most sensitive to CDF inhibition of amylase activity, the association of CDF and amylase does not have any effect on amylase catalytic activity. This conclusion is confirmed by the observation that 90 mM Tris has the same effect on PNPM activity of CF, H, and N saliva (Table 2). CDF has no detectable effect on either PNPM or Tris binding to α -amylase.

SALIVARY AMYLASE LEVELS

To further characterize CF saliva samples, the amylase starch activity and protein concentration in CF, H, and N saliva were compared (Table 3). Although the mean amylase and protein concentrations for CF saliva were found to be higher than the means of the normal saliva ($P < 0.001$ and $P < 0.005$, respectively), the specific activities were not significantly different (by Student *t*-test).

The distribution of values for amylase and protein concentration in CF, H, and N saliva is indicated in Figure 1. The large range of values is not due to fluctuations in composition of individuals' saliva. Up to eight determinations were made on repeated samples from 17 individuals. Samples were taken during different visits to the clinic or laboratory. Repeated determinations varied by less than 1 SD of the respective genotype mean.

COMPARISON BY AGE AND SEX

The amylase specific activity for normal samples appears to peak in preadolescence, and then drops sharply during adolescence (linear regression vs. age, $P = 0.032$) (Fig. 2A). The heterozygote samples, from a necessarily older population, are similar to normal adults. CF samples show a drop similar to the normal subjects as age increases from 8–13 years, but have increased activity in the 14–24 year age group. This increase is due entirely to the fact that teenage CF females have elevated amylase specific activity compared to all other groups ($P < 0.005$) (see Fig. 3, A and D).

Salivary protein concentration appears to be increased for the

Table 3. Amylase activity and protein concentration of whole saliva¹

	n	Mean age (range)	Amylase conc, ¹ starch units/ml $\pm$ SD	Protein conc, ² mg/ml $\pm$ SD	Amylase sp act, units/mg $\pm$ SD
CF	25	17.4 (05-30)	56 $\pm$ 37 ³	0.74 $\pm$ 0.64 ⁴	106 $\pm$ 72
Male	14	17.7 (05-30)	51 $\pm$ 40 ⁴	0.78 $\pm$ 0.76	98 $\pm$ 77
Female	11	16.9 (09-27)	64 $\pm$ 31 ⁵	0.68 $\pm$ 0.45	116 $\pm$ 64 ⁶
Heterozygote	24	36.5 (20-58)	40 $\pm$ 28	0.62 $\pm$ 0.39	79 $\pm$ 47
Male	5	37.8 (22-57)	17 $\pm$ 13 ^{7,8}	0.38 $\pm$ 0.31	84 $\pm$ 82
Female	19	35.2 (20-58)	47 $\pm$ 28 ⁸	0.68 $\pm$ 0.38 ⁷	78 $\pm$ 31 ⁶
Normal	42	17.7 (05-43)	32 $\pm$ 16 ³	0.48 $\pm$ 0.34 ⁴	97 $\pm$ 69
Male	24	17.8 (06-43)	31 $\pm$ 12 ^{4,7}	0.52 $\pm$ 0.35	109 $\pm$ 89
Female	18	16.4 (05-38)	34 $\pm$ 20 ⁵	0.43 $\pm$ 0.31 ⁷	97 $\pm$ 50

¹ Determined using starch assay. See *Materials and Methods*.² Determined using Miller's modification (20) of the Lowry procedure (16).³ CF vs. N, $P < 0.001$ ⁴ CF vs. N, $P < 0.05$ ⁵ CF vs. N, $P < 0.005$ ⁶ CF vs. H, $P < 0.05$ ⁷ H vs. N, $P < 0.05$ ⁸ H females vs. H males, $P < 0.05$.

Fig. 1. A: distribution of α -amylase concentration values for each genotype. Amylase activity was determined using the starch assay as described under *Materials and Methods*. B: protein concentration values by genotype. C: amylase specific activity values by genotype. The bars represent the mean $\pm$ SEM for each genotype.

CF samples in the 14-24 year age group (Fig. 2B). This increase is due to the increased salivary protein concentration of adolescent CF males compared to all other groups ($P < 0.005$) (Fig. 3, B and E). Heterozygote mean values fall within the large normal range (Fig. 2B).

The greatest difference between CF and N samples is in the amylase concentration per ml saliva. Both male and female CF homozygotes have highly elevated salivary amylase concentration in the 14-24 year age group ($P < 0.001$) (Fig. 3, C and F). The normal samples show no significant variation over the age range studied (5-42 years, $P = 0.941$). The heterozygote sample values appear to increase with age (linear regression for heterozygote females vs. age, $P = 0.016$) but more H samples as well as N samples in the older age range are needed to determine the significance of the apparent increase.

AMYLASE HYPERSECRETORS

The elevation of mean CF amylase concentration (Table 3) and protein content can be attributed to a group of amylase hyperse-

cretors (Fig. 1). CF values are distributed in a bimodal fashion, such that the values are either in the normal range or are markedly elevated. Sixteen of 25 CF patients had amylase concentrations within two SDs of the N mean; the remaining 9 values were 3 or more SDs above the N mean (Fig. 1). Similarly, 19 of 25 CF protein values were in the range of the N mean $\pm$ 2 SD, whereas the other 6 values were higher than the maximum N values. The protein hypersecretors were also amylase hypersecretors, so that the specific activity of salivary amylase in CF patients was neither bimodal nor different from the N mean.

CF-PARENT CORRELATION

All nine amylase hypersecretors were found in the 14-24 year age group. However, three patients in this age group were not hypersecretors. To determine whether hypersecretion is related to hereditary factors or to the clinical status of the patient, CF values were correlated to parent values and to Schwachman scores (25). For 11 patient-parent pairs, patient and parent ages were strongly correlated: $P = 0.0017$. Amylase concentrations were marginally correlated ($P = 0.075$), but protein concentration and amylase specific activity were not correlated for the two groups.

Our patient population included one affected sibling pair. Both the 15-year-old female and her 12-year-old brother had normal amylase concentration (35 and 19 starch units/ml) and protein content (0.440 and 0.438 mg/ml). This along with the weak patient-parent correlation of amylase concentration is consistent with some genetic influence on hypersecretion. However, data on additional affected sibling pairs is needed to define the genetic relationship to hypersecretion.

SHWACHMAN SCORE CORRELATION

The clinical status of the CF patients (assessed by the Shwachman score (25)) correlated marginally ($P = 0.068$) with the amylase concentration. The protein and specific activity values did not correlate significantly with the clinical status of the patients.

As expected, the age of the patient and the Shwachman score were correlated ($P = 0.0011$). The regression of Shwachman score vs. age for males had a slope of one half that observed for the females: $y = 87 - 1.2x$ vs. $y = 89 - 2.0x$. The regression lines for the two sexes were different ($P = 0.029$). A sharper decline in female vs. male CF patients with age has been suggested previously (24, 31).

DISCUSSION

The CDF fraction isolated from CF and H saliva has been found to inhibit mammalian debranching enzyme (11). This activ-

ity of the CDF has allowed replacement of the unwieldy and erratic oyster gill bioassay for CDF activity (4) with a quantitative enzymatic assay for salivary CDF activity (11). Unfortunately, this procedure requires prior separation of the CDF fraction from the salivary amylase, since amylase destroys the debranching enzyme substrate. This separation is cumbersome and the stability of CDF under the conditions of separation is unknown. Therefore, this assay retains major shortcomings.

Ideally, an enzymatic assay for CDF would use patient materials directly. Since amylase is already present in saliva, and since it is closely associated with the salivary CDF (1, 8, 9, 11), the effect of CDF on this enzyme was studied. Unlike its action on the debranching enzyme, CDF was found to have no detectable effect on α -amylase even under reaction conditions designed for maximum competitive and noncompetitive inhibition. Neither the amylase catalytic activity nor its binding to Tris is altered by the presence of CDF.

The 1:1 dilution used may not be a sufficiently high concentration of CDF to affect α -amylase catalytic activity. The debranching enzyme is about 5 times more sensitive to Tris inhibition than is α -amylase ($K_i = 12$ mM (13) and $K_i = 59$ mM, respectively). The concentrated CDF fraction from larger quantities of saliva may yet be found to affect α -amylase activity. However, for direct measurements on CF body fluids, a more sensitive assay must be developed. Although the CDF and α -amylase are closely associated in CF saliva, their mode of interaction remains to be defined.

To provide information on the basis for elevated α -amylase levels in CF, the amylase activity and protein concentration of CF, H, and N saliva samples were compared as a function of age and sex. Our results (Table 1) indicate that mean salivary amylase concentration and protein content are in the order CF > H > N, with overlap of values among the groups.

The magnitude of the difference between CF and N values is

Fig. 2. Salivary amylase and protein content by age and genotype. Each point corresponds to the mean $\pm$ SEM of four or five individual values, plotted vs. their mean age. CF, $\bullet$ — $\bullet$; H, $\circ$ — $\circ$; N, $\blacktriangle$ — $\blacktriangle$. A: amylase specific activity; B: protein concentration; C: amylase concentration in starch units per ml.

Fig. 3. Comparison of male and female salivary composition for CF and N samples. Each point corresponds to the mean $\pm$ SEM for three to five individuals, plotted vs. their mean age. Males, $\bullet$ — $\bullet$; females, $\circ$ — $\circ$. A: amylase specific activity of CF samples; B: protein content of CF samples; C: amylase concentration of CF samples; D: amylase specific activity of N samples; E: protein content of N samples; F: amylase concentration of N samples.

age dependent (Fig. 2). This variation with age explains previously described differences between CF and N saliva (2, 3, 17). For example, Blomfield's group reported that amylase concentration was elevated in the parotid saliva of a group of 3- to 17-year-old CF patients (2). Some of Blomfield's patients were hypersecretors. The same group subsequently reported that amylase levels in 9- to 13-year-old CF patients were not significantly different from the N mean (3). We have found a sharp increase in CF whole saliva amylase concentration in patients between 14 and 24 years of age ($P < 0.005$). The cause for this difference between CF children, adolescents, and young adults remains to be determined. Normal saliva shows no significant change in amylase concentration from 5-42 years of age.

In contrast to the constant amylase concentration of normal saliva, the mean specific activity of normal saliva decreases dramatically with increasing age ($P = 0.032$) (Fig. 2). CF males show a similar decrease (Fig. 3) but CF females demonstrate an increase in specific activity which peaks at 17 years. This change with age may represent the functional development of the various salivary glands (26) and possible hormonal influences (*cf.* Ref. 26) which depend upon both age and sex. In any event, these data emphasize the need for age- and sex-matched controls in all studies of salivary composition.

The existence of a subgroup of amylase hypersecretors in the CF patient population is another example of the heterogeneity of this disease. The basis for hypersecretion of amylase in CF remains to be elucidated. Both hereditary and clinical factors are marginally correlated to the elevated amylase concentrations. The high amylase values suggest that the parotid gland may be secreting a higher proportion of the whole saliva than normal. One might invoke three hypotheses to account for this. First, the parotid

gland may be contributing a greater portion of the whole saliva because of plugging of the mucus-secreting glands. This type of plugging in the pancreas accounts for progressive loss of pancreatic exocrine function in CF (5, 31). Second, the parotid gland may hypersecrete in order to maintain the serum amylase levels. Serum pancreatic amylase is decreased in CF patients with pancreatic insufficiency yet the total serum amylase is maintained in the normal range by salivary isozymes (26, 29, 30). If this is the case, hypersecretion of amylase in CF saliva may prove to be a sensitive, indirect measure of deteriorating pancreatic exocrine function. Third, amylase hypersecretion may be more directly related to a primary defect in the secretory mechanism in CF. Amylase release is mediated by intra- and extracellular Na^+ and Ca^{2+} (22). Alterations in electrolyte transport or permeability could affect secretory function. Amylase secretion is altered in cystic fibrosis. Its role in the pathophysiology of the disease remains to be determined.

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