Comparative Studies on Salivary Kallikrein from Cystic Fibrosis Patients and Controls

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ABSTRACT. Kallikrein (EC 3.4.21.8) has been purified from the saliva of cystic fibrosis (CF) patients and from healthy individuals. The yields of enzyme are the same for both kinds of saliva. The CF and normal kallikrein have similar physical-chemical properties, such as amino acid composition, electrophoretic mobilities in polyacrylamide gels when different conditions are used, intrinsic fluorescence, and circular dichroism. The enzymes have no α-helix structure but large amounts of pleated sheet structure. CF and normal salivary kallikrein have also similar enzymatic properties, for example both enzymes show maximum activity at pH 8.2. An identical value of $K_m = 0.4$ mM has been found for both enzymes with N-benzoyl arginine ethyl ester as substrate, despite the fact that the kallikreins are inhibited by high substrate concentrations. The results of this investigation show that the salivary kallikrein in CF is normal and that it has normal activity. This leads us to suggest that the salivary kallikrein is not the cause of the observed abnormalities in CF saliva. (*Pediatr Res* 19: 938–943, 1985)

Abbreviations

BAEE, N-benzoyl arginine ethyl ester
BPTI, bovine pancreas trypsin inhibitor
CD, circular dichroism
CF, cystic fibrosis
HPLC, high performance liquid chromatography
pI, isoelectric point
SDS, sodium dodecylsulfate

Abnormal secretions have been recognized for some time as one of the basic abnormalities in patients with CF (1, 2), for example the elevation of sodium levels in secretions is a consistent finding in CF (3).

Salivary glands secrete an isotonic solution of electrolytes and macromolecules, but during its passage through the glandular ducts ions are reabsorbed and the solution becomes hypotonic. Dann and Blau (4) suggested in 1978 that the reabsorption of ions in glandular ducts is controlled by arginine esterases which are present in the glands. At the same time they proposed that abnormal electrolyte concentrations in CF secretions are caused by a deficiency in either the glandular kallikrein, which itself has arginine esterase activity, or in some other arginine esterase. In 1981 Hallinan (5) expanded this hypothesis and proposed that CF could be due to a possible defect in an intracellular enzyme, which carries out an as yet unknown but functionally important

posttranslational modification to the arginine esterases, for example a glycosylation or phosphorylation.

Catanzaro *et al.* (6) demonstrated in 1978 that kallikrein is involved in the control of Na^+, K^+, and H_2O secretions in the saliva of the rat. Subsequently it has been shown that kinins, which are small peptides that are produced by kallikrein through cleavage of kininogens, are directly involved in the inhibition of electrolyte absorption in guinea-pig ileum (7). Because of these findings it was decided to study the kallikreins in normal and CF saliva and to investigate whether the hypothesis of Dann and Blau (4) or that of Hallinan (5) is correct.

The isolation of human salivary kallikrein has been reported by Fujimoto *et al.* (8) and by Modeer (9). More recently Hoffman *et al.* (10) isolated this enzyme by selective catheterization of glandular ducts while Wong *et al.* (11) purified the enzyme from parotid saliva. However, significant differences have been reported in the properties of the purified salivary kallikreins, for example in isoelectric focusing and molecular weights. We have therefore recently developed a new method to purify kallikrein from human whole saliva. Several properties of the purified kallikrein from the saliva of CF patients will now be reported together with those of the normal kallikrein that have not been previously reported.

MATERIALS AND METHODS

Whole saliva, stimulated by chewing dental wax (Cybron/Kerr) was obtained from both male and female volunteers. The CF patients were in the age group between 18 and 25 yr. They were all diagnosed at a young age with the sweat-test and they have been under regular observation in the CF-clinic at the I.W.K. Hospital for Children in Halifax, N.S. Canada, which is under the direction of Dr. C. T. Gillespie. The patients were mostly university students and were considered to be in good condition. They produced each about 150 ml saliva per turn and the saliva of three to four patients were pooled to give a volume of about 500 ml. The pooled samples consistently contained higher concentrations of protein, carbohydrate, and nondialyzable compounds, than normal saliva.

A Spherogel-TSK 3000SW column (7.5 × 300 mm) was purchased from Beckman Instruments for use in HPLC.

The synthetic substrate BAEE and trypsin inhibitors were obtained from Sigma.

The purification procedure for salivary kallikrein is described elsewhere (12). The same procedure was used for normal and CF saliva.

Protein determinations were done with Coomassie Blue by the Bio-Rad method (13) and a protein standard, purchased from Bio-Rad, was included in each determination. Enzymatic activity was determined with BAEE as substrate and the reaction was monitored at 253 nm (14) with a Cary 14 recording spectrophotometer.

Gel electrophoresis has been done as described elsewhere (12) and the molecular weights of the salivary kallikreins were calcu-

lated from their mobilities in the SDS containing gels using a reference mixture of standard proteins (obtained from Pharmacia). Isoelectric focusing was done using a LKB 8101 column (LKB, Sweden). The pH gradients were formed with Pharmalyte pH 2.5–5.0 in sucrose density gradients.

Amino acid analyses were done with a Beckman model 6300 analyser after hydrolysis of the enzymes in 6 M HCl. Separate samples were oxidized with performic acid at 2° C for 4 h and then analyzed for cysteic acid and methionine sulfone (15). Tryptophan was determined by the method of Goodwin and Morton (16).

Fluorescence measurements were made with an Aminco-Bowman spectrofluorometer at room temperature, using a 3 mm^2 cuvette which requires only 100 μl of solution. The spectra were corrected as outlined by Melhuish (17) and a horizontally oriented polarizer was used in the excitation beam to reduce scattering (18). The corrected intrinsic fluorescence spectra were analyzed to determine the number of independent fluorescent components (19).

Circular dichroism spectra were measured at wavelengths below 240 nm with a Cary 6001 instrument. The data were converted to deg·cm^2·dmol^{-1} (20) using a mean residue weight of 107 as calculated from the amino acid composition. The secondary structures were computed using the method of Chang *et al.* (20), who also provided us with the four reference spectra, *e.g.* those for α-helix, pleated sheet, β-turns, and unordered structures. The following constraints were used: (1) none of the aforementioned structures could be present in the protein at more than 100% or less than 0%, and (2) the sum of the four structures must be 100%.

RESULTS

The flow chart illustrated in Figure 1 summarizes the procedure for the purification of a salivary BAEE-esterase from either CF patients or normal individuals. It has previously been shown that a partially purified BAEE esterase from normal saliva produces kinins in acid-treated dog plasma (21). The same enzyme also reduces the blood pressure of a rabbit when injected intravenously. This enzyme therefore seems to belong to the glandular kallikreins.

Affinity chromatography on BPTI-Sepharose gives the greatest increase in purity in a single step, but it fails to remove an inactive protein component from the enzyme. Complete purity of the enzyme is not obtained until after HPLC on a Spherogel-TSK 3000 SW column (see Fig. 2). HPLC separates two protein components, but only the material that elutes between 21 and 24 min contains enzymatic activity, the other component is completely inactive. The inactive component is present in normal and in CF saliva and appears to bind to the kallikrein because it does not itself show affinity for the BPTI-Sepharose column.

The saliva of CF patients contains similar BAEE esterase activity as saliva of normal controls. Examples of the purification of kallikrein from saliva are shown in Table 1. Understandably the data fluctuate between different batches of saliva but the
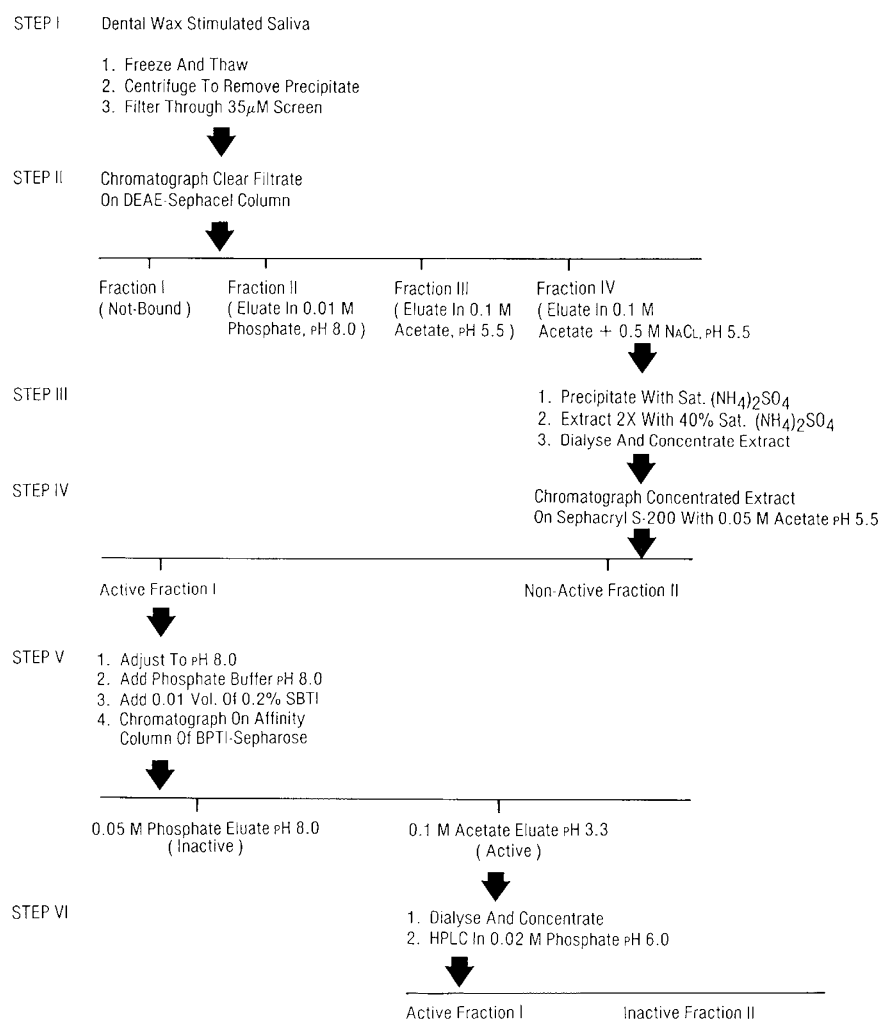

Fig. 1. A flow chart illustrating the purification of kallikrein from whole saliva.

results shown in Table 1 are quite typical. It can be seen that the final purification of the CF kallikrein and its activity are the same as that for the normal enzyme, and this purification as well as the yield does not vary more than 10–20% from batch to batch.

Polyacrylamide gel electrophoresis experiments at pH 9.5 and at pH 8.2 in the presence of SDS, show that the CF enzyme has normal mobilities (see Fig. 3). The molecular weights have been determined from the mobilities in the SDS containing gel and a value of 40,000 has been calculated for both normal and CF salivary kallikrein.

The amino acid compositions of the enzymes are shown in Table 2. The compositions are very similar. Both enzymes contain a large number of aspartic and glutamic acid residues and only small numbers of the various basic residues. The pI values of the salivary kallikreins have also been determined by isoelectric focusing and as anticipated from the amino acid compositions, low values, pI = 4.00 ± 0.08, have been found. The methionine and cysteine contents of both enzymes are very low and an accurate determination of these residues proved to be almost impossible. Slight discrepancies have been observed for the proline and valine residues but otherwise the amino acid contents are the same within 1 SD ($n = 4$).

Normal and CF salivary kallikrein each contain 1 mol percent of tryptophan whose presence has also been demonstrated by measuring the intrinsic fluorescence spectra. The corrected excitation spectra of both enzymes at pH 7.0 show a maximum at 282 nm while the corrected emission spectra show a broad peak centered at 338 ± 1 nm (see Fig. 4). The fluorescence intensities at 338 nm are the same for normal and CF enzyme solutions after correcting for the difference in absorbances at 275 nm. An algebraic method was used to analyze the emission spectra obtain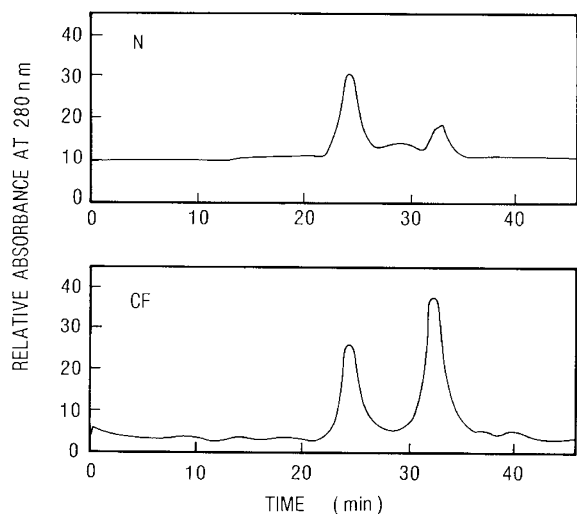ed at four different wavelengths of excitation, and it was found that two independent groups contribute to the intrinsic fluorescence of normal and CF kallikrein. These groups are presumed to be tyrosine and tryptophan.

The CD spectra of the salivary kallikreins are shown in Figure 5. Both spectra show a maximum negative ellipticity at 197 ± 1 nm with values of $[\theta] = -12,000 \pm 1,300 \text{ deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. The curves were analyzed with a computer using four basic reference spectra, and the results are shown in Table 3. It can be seen from Table 3 that the salivary kallikreins have no α-helix structure. On the other hand, the enzymes have substantial amounts of pleated sheet structure. The data of Table 3 have been used to calculate the best fit of the observed spectrum for normal kallikrein (see Fig. 5). This computed curve resembles, but does not exactly fit, the observed spectrum.

The enzymatic properties of the CF salivary kallikrein have been investigated and compared with those of the enzyme obtained from control saliva. Neither enzyme cleaves azocasein nor are they inhibited by soybean or lima bean trypsin inhibitors. On the other hand, both enzymes are inhibited by BPTI and also by di-isopropyl fluorophosphate. As shown in Table 4 the enzymes also reduce the blood pressure of a rabbit when injected intravenously. When 10 micrograms protein, corresponding to 20–25 ml saliva, is injected the rabbit's blood pressure is reduced by about 10 mm Hg.

The pH rate profiles for normal and CF salivary kallikreins are shown in Figure 6. It can be seen that neither enzyme cleaves BAEE at pH values below 5.0. The activities increase rapidly above pH 6.0 and reach maximum values at pH 8.2 ± 0.2. At high pH the enzymes remain fairly active and at pH = 10.0, for example, the activities are still about 80% of the observed maxima.

When the concentration of the substrate BAEE is increased to in excess of 1 mM, the reaction velocities begin to decrease. This substrate inhibition is observed for both CF and normal salivary kallikrein, and with freshly recrystallized BAEE. Figure 7 illustrates the double reciprocal plots of velocity *versus* BAEE concentration for the enzymes. These Lineweaver-Burk plots are linear if the data points at higher BAEE concentrations are ignored. By extrapolating these linear parts of the plots one obtains the apparent values for Km and Vmax which are shown in Table 4. From this Table 4 it is evident that the CF and normal salivary kallikreins have very similar kinetic parameters.

DISCUSSION

A BAEE-esterase has been purified from whole saliva from CF patients and controls. The demonstrated activities and biological effect of this enzyme and also the effects of trypsin inhibitors on the esterase activity, made us name this enzyme a kallikrein.

The batches of CF saliva showed often a higher viscosity than the normal batches but this did not appear to affect the purification procedure. The yield and the purification factor indicate that CF saliva contains a normal level of kallikrein which amounts to 0.4–0.5 μg/ml. The most important purification step is the affinity chromatography on a BPTI-Sepharose column. However, not all the inactive protein components are removed during this step, because an inactive protein, which is rather

Fig. 2. HPLC of normal and CF kallikrein on a Spherogel-TSK 3000 SW column (7.5 × 300 mm). The flow rate was 0.6 ml of 0.01 M phosphate pH 5.6 per min at room temperature.

Table 1. *The purification of salivary kallikreins*

Preparation	Protein (mg)*		Activity†		Yield (%)		Purification	
	N	CF	N	CF	N	CF	N	CF
Whole saliva, centrifuged and filtered	605.0	700.0	1.78	1.64	100	100	1	1
DEAE-Sephacel, fraction IV	59.0	66.0	16.0	16.4	88	94	9	10
Sephacryl S-200	12.0	13.7	60.5	52.5	67	63	34	32
BPTI-affinity	0.512	0.525	987.0	1017.0	47	47	554	620
HPLC	0.066	0.053	4880.0	4760.0	30	22	2742	2902

* The purification was started with 500 ml. N, normal.

† Activity in nmol/min/mg protein. Protein was determined with the Bio-Rad method and the substrate was BAEE.
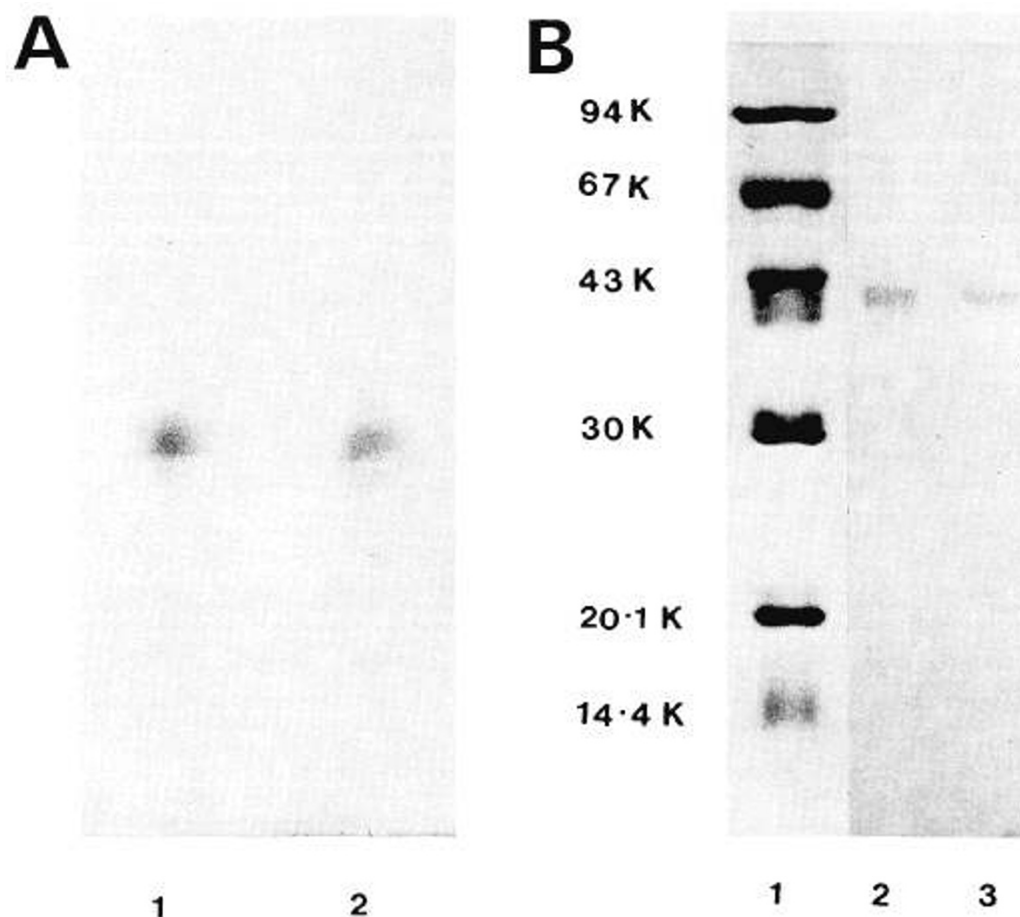
Fig. 3. Polyacrylamide gel electrophoresis of normal and CF salivary kallikreins at pH 9.5 (*A*) and at pH 8.2 in the presence of SDS (*B*). Normal enzyme in channels A1 and B2, CF enzyme in channels A2 and B3, and a mixture of callibrating proteins in channel B1.

Table 2. *Amino acid compositions of the salivary kallikreins*

	CF enzyme*	Normal enzyme†
Aspartic acid	10.8 ± 0.5	10.8 ± 0.7
Threonine	5.3 ± 0.3	5.2 ± 0.2
Serine	11.5 ± 0.8	11.1 ± 0.7
Glutamic acid	13.6 ± 1.7	15.3 ± 2.1
Proline	4.7 ± 0.7	5.9 ± 0.6
Glycine	12.4 ± 0.8	13.3 ± 0.8
Alanine	6.6 ± 0.4	6.5 ± 0.3
Valine	6.3 ± 0.4	5.4 ± 0.1
Cysteine	0.7 ± 0.4	0.4 ± 0.3
Methionine	1.1 ± 0.4	1.1 ± 0.4
Isoleucine	3.0 ± 0.3	2.6 ± 0.2
Leucine	7.4 ± 0.5	7.6 ± 0.3
Tyrosine	2.4 ± 0.4	2.3 ± 0.2
Phenylalanine	3.7 ± 0.3	3.4 ± 0.1
Lysine	3.1 ± 0.5	3.3 ± 0.3
Histidine	2.9 ± 0.4	2.6 ± 0.3
Arginine	3.3 ± 0.6	2.8 ± 0.4
Tryptophan	1.0	1.0

* Mean ± 1 SEM of three determinations.

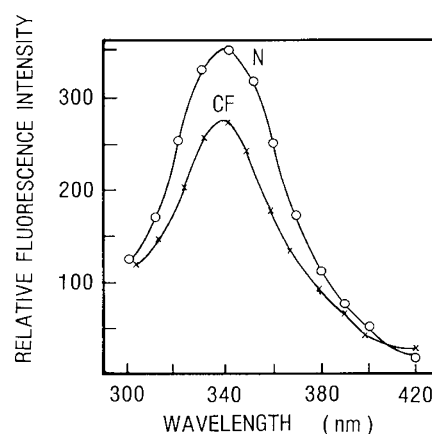† The mean value ± 1 SEM of four determinations, see Reference 12.

Fig. 4. Corrected fluorescence spectra of normal and CF kallikreins in 0.02 M phosphate buffer pH 7.0. The wavelength of excitation was 282 nm. The fluorescence intensities at 338 nm are directly proportional to the absorbances of enzymes which were 0.213 for normal enzyme and 0.164 for CF enzyme at 275 nm.

proline-rich, is still present. HPLC has shown that this is the case for normal as well as CF saliva. Since this inactive contaminant does not bind to the BPTI-Sepharose column it is assumed to be associated with the salivary kallikreins, from which it dissociates at weekly acidic pH.

The enzymes purified from CF and normal saliva show identical electrophoretic mobilities in polyacrylamide gels at pH 9.5 and in gels containing SDS at pH 8.2. Both enzymes show only one band in either gel which proves that they are pure. The identical mobilities of the enzymes in the presence of SDS suggests that the CF kallikrein has the same molecular weight of 40,000 as the normal enzyme. The latter's molecular weight has previously been confirmed by sedimentation equilibrium (12).

The amino acid compositions of CF and normal kallikreins are very similar. Both enzymes have high contents of acid residues while the basic amino acid residues are only small in
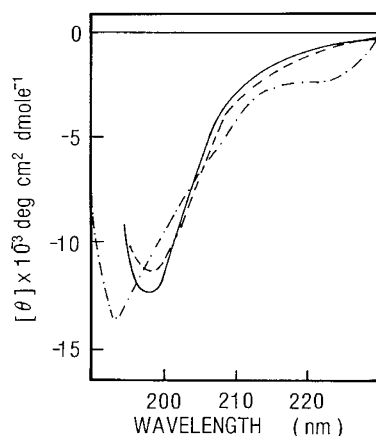
Fig. 5. The CD spectra for normal (——) and CF (– – –) salivary kallikreins in 0.02 M phosphate buffer, pH 6.0. The data for normal enzyme, given in Table III, were used to plot the curve (–·–·–) which represents the closest fit.

Table 3. *Secondary structures of salivary kallikreins*

Preparations*	α-Helix†	Pleated sheet	β-Turn	Unordered
Normal enzyme	0	41	15	44
Normal enzyme in SDS	0	51	8	41
CF enzyme	0	38	13	49

* Enzyme solutions in 0.02 M phosphate buffer pH 6.0, and in phosphate buffer with 0.3% (w/v) SDS.

† Data are in percents and have been computed from the curves shown in Figure 5.

Table 4. *Enzymatic properties of salivary kallikreins*

	CF enzyme	Normal enzyme
Optimum activity*	pH 8.0–8.5	pH 8.0–8.5
K_m	0.4 mM	0.4 mM
V_{max}	20 μmol/min/mg	21 μmol/min/mg
k_{cat}	13 s^{-1}	14 s^{-1}
Azocasein (1 mg/ml)	Not digested	Not digested
Soybean and lima bean trypsin inhibitors	Inhibition <2%	Inhibition <2%
Pancreatic trypsin inhibitors	Inhibition >98%	Inhibition >98%
Diisopropyl fluorophosphate† (10^{-5} M)	Inhibition >80%	Inhibition >80%
Drop in blood pressure when 10 μg enzyme is intravenously injected in a rabbit (mm Hg)‡	10 mm	10 mm

* BAEE was used for substrate. Activities were measured with about 5–10 μg enzyme per millimeter.

† Diisopropyl fluorophosphate is less effective for kallikrein than for trypsin. At pH 8.0 the activity of kallikrein is more quickly restored than that of trypsin when the solutions are dialyzed at pH 8.0.

‡ This test was carried out as described in Reference 21.

numbers. Because of the uneven contents of the residues in the proteins the analyses are less accurate than is normally the case, and especially the sulfur-containing amino acids were difficult to determine. The amino acid compositions indicate low isoelectric points for the salivary kallikreins. This has been confirmed by isoelectric focusing of the normal and CF kallikreins which gave values of pI = 4.00 ± 0.08 for both. Isoelectric focusing has also confirmed that the enzymes are pure.

The corrected intrinsic fluorescence spectra of the CF and normal salivary kallikreins are identical. Although the spectra show only one broad peak, calculations have shown that two
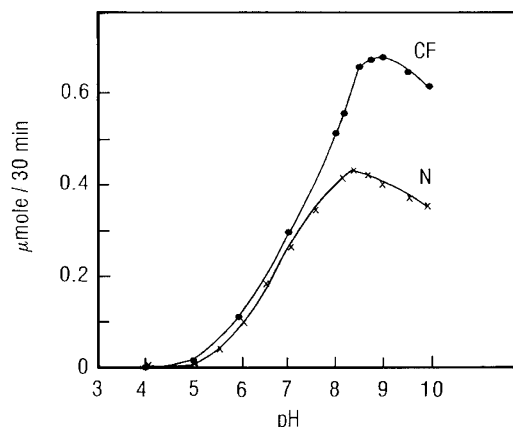

Fig. 6. The pH rate profiles for normal and CF salivary kallikreins. Aliquots of the enzymes were added to 0.6 mM BAEE in 0.1 M phosphate buffer pH 5.0 to 8.0 and 0.1 M Tris-HCl buffers pH 8.0 to 10.0. The reactions were followed at 37° C with a Cary model 14 recording spectrophotometer.
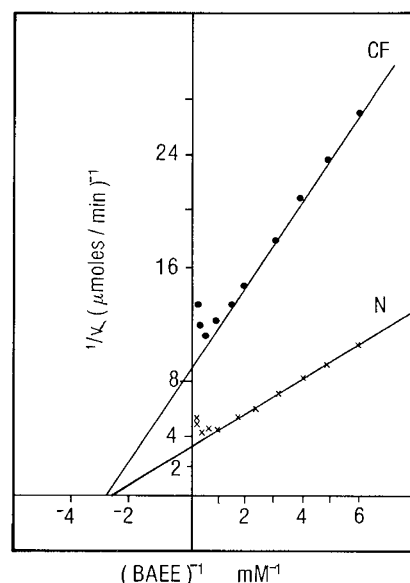

Fig. 7. Lineweaver-Burk plots for the hydrolysis of BAEE in 0.1 M Tris-HCl pH 8.0 at 37° C.

different fluorophores, presumably tyrosine and tryptophan, contribute to the emissions. The quantum yield of the CF enzyme is exactly the same as that of the normal salivary kallikrein.

The CD spectra of the enzymes are rather unusual because they show very little ellipticity at wavelengths above 210 nm. This indicates very little α-helix structure in the enzymes. Even at the extrema, at 197 ± 1 nm, the observed ellipticities are low. The experimental error in the measured ellipticities is therefore between 10 and 15%. The CD data have been analyzed by the method of Chang *et al.* (20) and the results indicate that the enzymes have indeed no α-helical structure. Because of the fact that the three remaining basis spectra (thus those for the pleated sheet, β-turns, and unordered polypeptide chains) are less reliable, the data given in Table 3 should be interpreted with caution, especially since the calculated curve does not exactly fit the observed spectra. However, despite these reservations it is clear that the pleated sheet is the major structure in CF as well as in normal salivary kallikrein.

The enzymatic properties of the CF salivary kallikrein are the same as those of the normal enzyme. Although the kallikreins are likely to be serine proteases, as demonstrated by their inhibitions by diisopropyl fluorophosphate, no evidence has yet been

found for an acyl-enzyme intermediate. The initial hydrolysis of the artificial substrate BAEE is linear with time. The pH rate profiles are not exactly bell-shaped as they only show a minor drop in activity at high pH values. Substrate inhibition is observed with BAEE at pH 8.2 even if carefully purified substrate is used. The inhibitions at high BAEE concentrations are identical for the normal and CF enzyme. Since we have observed that polylysine also lowers the enzymatic activity it is believed that the substrate inhibition is caused by interactions on secondary binding sites. The presence of such sites could explain the fact that the pH rate profiles are not bell-shaped, because a decrease in BAEE affinity at these sites would obscure the reduction in arginine esterase activity at high pH. Regardless of the mechanism of the substrate inhibition its effect seems to be small at low concentrations of BAEE. Thus linear Lineweaver-Burk plots are still observed at low substrate concentrations. The kinetic parameters calculated from these plots for normal and CF kallikreins are almost identical, despite the fact that long extrapolations are required which means that the results could be somewhat less reliable.

Thus our study shows that salivary kallikrein is present at the same level in CF saliva as in normal saliva and that it has very similar physicochemical properties. CD measurements attest to identical secondary structures while the intrinsic fluorescence indicates similar conformations. Furthermore the enzymatic properties of the CF salivary kallikrein are the same as those of the normal enzyme. It is therefore concluded that the salivary kallikrein in CF is not defect and that its activity is quite normal.

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