

PARTIAL PURIFICATION OF AMYLASE FROM HUMAN MIXED SALIVA (I)

by

Choi K.B., Han S.B., Lee H.C., Myoung K.S. and Chung T.Y.

Department of Dental Biochemistry

School of Dentistry, Seoul National University.

타액 Amylase의 부분 정제 (I)

서울대학교 치과대학 구강생화학교실

최근배 · 한수부 · 이해철 · 명광식 · 정태영

.....>국문초록<.....

사람唾液의 Amylase 部分 精製를 爲하여 各 %의 Ammonium Sulfate 包和 劃分을 얻은 다음 Calcium phosphate gel로 處理하고 各 濃度의 Na_2HPO_4 로 流出시킨 一次實驗結果는 다음과 같다.

1. Ammonium Sulfate 包和 劃分中 50-70% 包和 劃分의 Amylase 活性이 가장 높았다.
2. Amylase 比活性은 0.02M Na_2HPO_4 로 流出한 劃分에서 가장 높았다.

INTRODUCTION

The organic components in saliva have been studied for many years, yet knowledge of them remain fragmentary. But a few investigators have concentrated their efforts on the identification and characterization of those substances that held particular interest for them. The first through investigation on the nature of the proteins from saliva were carried out by means of free boundary electrophoresis¹⁾ and electrophoresis on filter paper. The information supplied by these studies has been supplemented by immunological data, particularly those provided by immunoelectrophoresis.²⁾ Chromatography and gel filtration has also occasionally been used to characterize human salivary protein.^{3,4)}

Amylase is the best studied of the salivary proteins. Knowledge of the salivary amylase is based largely on examination of preparations prepared from whole human saliva. Purification procedures and properties have been described and discussed by several workers.^{5,6,7)}

The authors are also interested in the purification and characterization of salivary

enzymes. This report described our first step in attempting to elucidate the properties of salivary enzymes, namely the purification of amylase by fractionation with saturated ammonium sulfate, adsorption in calcium phosphate gel and elution with Na_2HPO_4 increasing its molarity.

MATERIAL and METHOD

Collection of saliva: Salivary flow was stimulated by the subject chewing gum and saliva was collected in polyethylene tube immersed in an ice bath. Oral debris was removed by centrifugation at 10,600g and 4°C. The supernatant so obtained is referred to as whole saliva in this paper.

Assay of salivary amylase: Amylase activity was measured colorimetrically using dinitrosalicylic acid,⁷⁾ for determination of the liberated reducing sugar during incubation. The reaction mixture contained 0.2 ml. of enzyme solution, 1.0 ml. of substrate (1.0% starch solution), and 0.8 ml. of phosphate buffer (pH 6.9, 0.02 M containing 0.06 M NaCl) in total volume of 2.0 ml., and incubated at 37°C for 6 minutes. The enzyme reaction was terminated by addition of 2.0 ml of 3,5-dinitrosalicylic acid reagent. The tube containing this mixture was heated for 5 minutes in boiling water and then cooled in running tap water. After addition of 10 ml. of distilled water, the optical density was determined photometrically at 470 m μ wave length. The blank was prepared in the same manner without enzyme. A standard curve relating maltose concentration to the color reactions was obtained by reacting 2.0 ml samples of maltose solutions (concentrations of 0.1-1.0 mg/ml.) with 2.0 ml of 3,5-dinitrosalicylic acid reagent. One amylase unit was defined as the amount of reducing sugar, liberated after 6 min. reaction of pH 6.9 at 37°C, which is equivalent to 1 mg. of maltose.

Protein determination: The protein content was monitored by the method of Lowry *et al.*⁸⁾ in order to obtain specific activity of the enzyme preparations. Bovine serum albumin (Nutritional Biochemical Corp.) was used as standard determining the nitrogen content of it by kjeldahlometry. The specific activity of the enzyme preparations were thus expressed as units per mg. protein content of them.

Fractionation with saturated ammonium sulfate: The whole saliva, obtained from human mixed saliva, was saturated with ammonium sulfate, salting out the 0-30%, 30-50%, 50-70%, 70-80% saturated fraction. The precipitate, collected by centrifugation, was dissolved in 5 ml. of phosphate buffer used above, and then dialyzed overnight against the same buffer. Amylase activity was assayed in each dialyzate, and protein content was also determined. The dialyzed solution, which is the highest in activity of amylase, was treated batchwise with an amount of calcium phosphate gel,⁹⁾

Elution with Na_2HPO_4 increasing its molarity: The adsorbed protein in calcium phosphate gel was eluted with 0.01 M, 0.02 M, 0.05 M, 0.1 M and 0.5 M Na_2HPO_4 successively in order. The calcium phosphate gel elute in each concentration of Na_2HPO_4 was dialyzed in the same way as described above. The final dialyzed solution was evaluated in amylase activity and protein content.

RESULT and DISCUSSION

Amylase is a protein of molecular weight about 50,000. It contains one gram of calcium per mole and is inactive when this is removed.¹⁰⁾ It requires chloride for activation, although some other anions can substitute for it.

As tabulated in Table I., the total activity of 257.52 units observed in whole saliva supernatant. Comparing the activities in the whole saliva and the ammonium sulfate saturated fractions, there is a great loss of activity, amounting as much as 106.4 units (40.1% of the original), which casts a doubt to the authors whether this enzyme is inactivated through fractionation or not.

Table I. Amylase activity of ammonium sulfate saturated fraction from mixed saliva.

Preparation steps	Total activity	Total protein	Specific activity	Yield(%)
Whole saliva	257.52	1.99	130.3	100.0
0-30%	36.70	0.26	142.1	14.2
30-50%	41.05	0.47	87.9	15.9
50-70%	45.70	0.31	148.9	18.8
70-90%	27.99	0.32	87.7	10.8

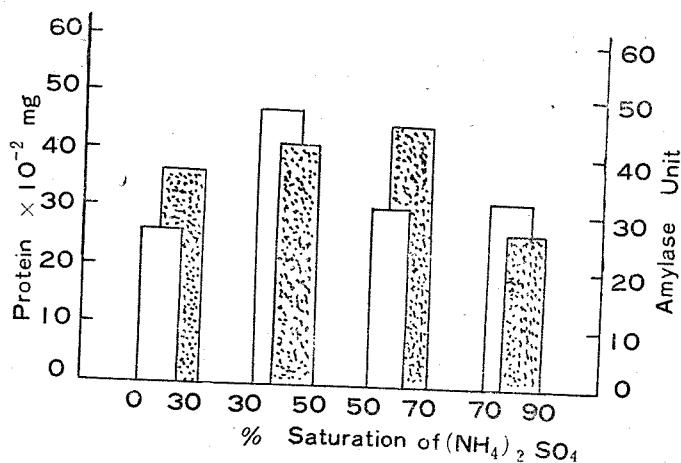


Fig. 1. A histogram showing the protein content and amylase activity in the 4 fractions obtained with ammonium sulfate solution.

As revealed in Fig.1, increasing the ionic strength of ammonium sulfate, 50-70% saturation is the richest recovery in amylase activity, The 0-30% of saturation precipitated a protein fraction 36.7, 30-50% of saturation 41.05, 50-70% of saturation 45.70, and 70-90% of saturation 27.99 units activity whereas the bulk of protein was precipitated in the 30-50% saturated fraction.

The 50–70% ammonium sulfate fraction was utilized, adsorbed to calcium phosphate gel, and eluted with Na_2HPO_4 increasing its molarity. The yield and potency of amylase activity through elution with increasing molarity of Na_2HPO_4 was tabulated in Table II.

Table II. Yield and potency of amylase after being adsorbed to the calcium phosphate gel. 50–70% saturated fraction of supernatant with $(\text{NH}_4)_2\text{SO}_4$ was adsorbed to the gel and eluted with Na_2HPO_4 increasing its molarity.

	Total activity	Total protein	Specific activity	Yield(%)
50–70% $(\text{NH}_4)_2\text{SO}_4$ precipitation	45.70	0,307	148.9	100.0
0.01 M Na_2HPO_4	10.00	0.048	210.0	21.8
0.02 M Na_2HPO_4	6.74	0.027	249.6	14.7
0.05 M Na_2HPO_4	5.93	0.033	179.7	12.9
0.10 M Na_2HPO_4	4.00	0.037	108.1	8.7
0.50 M Na_2HPO_4	2.25	0.030	75.0	4.9

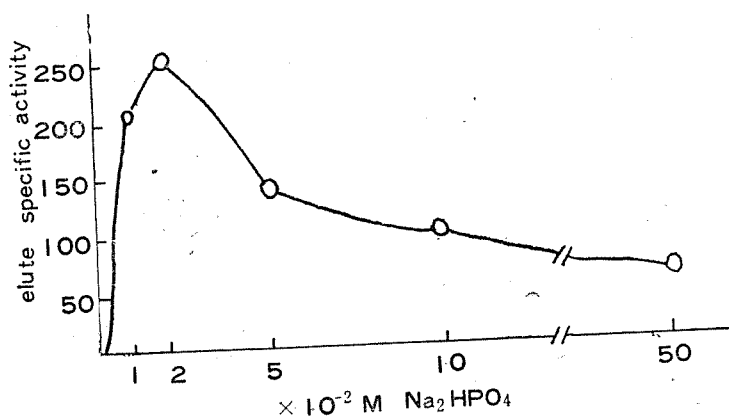


Fig. 2. An elution profile of the amylase after being adsorbed to the calcium phosphate gel. 50–70% saturated fraction of supernatant with $(\text{NH}_4)_2\text{SO}_4$ was adsorbed to the gel and eluted with Na_2HPO_4 increasing its molarity.

The elution profile, as in Fig. 2., showed that the elute with 0.02M Na_2HPO_4 is the highest in amylase specific activity (249.6 Unit). And therefore for the rich recovery of original activity, 0.02 M Na_2HPO_4 should be used in elution. Furthermore, if the fractionation with 50–70% saturation of ammonium sulfate is repeated, it is possible to purify highly.

Therefore, in the further study for the purification of amylase the authors decided to use 50–70% saturated fraction of supernatant with ammonium sulfate and let adsorbed

protein in calcium phosphate gel to elute with 0.02 M Na_2HPO_4 .

CONCLUSION

A preliminary study was performed to purify partially amylase from whole human saliva through ammonium sulfate fractionation, treatment with calcium phosphate gel and elution with Na_2HPO_4 increasing its molarity, and followed the result as below.

1. 50—70% ammonium sulfate saturated fraction is the richest in recovery of amylase
2. Elute with 0.02 M Na_2HPO_4 is the highest in amylase specific activity

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