# Effects of Sonic Oscillation on Taka-Amylase A and Its Derivatives.

## I. Degradation of Taka-Amylase A by Sonic Oscillation.

By Ikunoshin Kato

# Effects of Sonic Oscillation on Taka-amylase A and Its Derivatives.

## I. Degradation of Taka-amylase A by Sonic Oscillation

One of the most interesting problems in the studies on biologically active macromolecules, will be to elucidate the relationshpp between function and structure, and many efforts have been thus farmade along this line. One of the ways for this purpose is to isolate an artificially modified derivative of an active macromolecule and to compare its function and structure. The attempts to isolta an active derivative of enzyme have been performed by many authors ( 1 - 4 ).

In the studies on Taka-amylase A ( TAA ), the similar experiments have been successfully achieved by Toda et al. ( 5 - 7 ). Itis , however, obvious that structure and characters of an active fragment depend on the applied method for degradation. The general method for protein degradation is to use a specific proteolytic enzyme, but ordinarily native globular proteins are resistant to its action. TAA is found to be not an exception ( 8 ). Therfore Toda et al. proteolytically degraded TAA after converting it to its phenylazobenzoyl

derivative ( 9 ). In the present studies, we intended to modify the intact TAA by a non-proteolytic method without any pre-treatment.

Already, there are a few non-enzymatic methods, chemical and physical. For example, the former is to use a specific chemical reagent and the later irradiation of high energy beams. But those methods usually accompany with mortal damages for the biological function of enzymes and could not be applied for obtainning active derivatives.

In 1945, it was reported that sonic wave has denaturating action for protein molecules ( 10 ), but its effects on primary structure of the proteins and their biological functions were not examined. In turn, Schmid has found that polystylene of molecular weight 550,000 - 850,000 were degraded to of molecular weight 190,000 ( 11 ). These studies suggest that sonic wave has degradative action to chemical bonds.

Thus we applied mechanochemical force of sonic oscillation for the degradation of the intact TAA and have obtained two kinds of active derivatives.

This paper presents the effects of sonic oscillation on the primary structures of the intact TAA and of the reduced-

carboxymethylated TAA ( R-CM-TAA ), and their biological activities. The biologically active products were isolated chromatographycally.

## METHODS AND MATERIALS.

Taka-Amylase A.- - - Crystalline Taka-amylase A was prepared from " Takadiastase Sankyo " by the method of Akabori et al. ( 12 ) and recrystallized two or three times from aqueous acetone. The crystalline TAA was further purified by DEAE-cellulose column chromatography ( 13 ). The purified TAA was dialyzed against a large volume of deionized water and stocked after lyophilization. The molecular weight of TAA was assumed as 51,000 ( 14 ).

Reduction and Carboxymethylation of Taka-amylase A ( R-CM-TAA )

The reduction of 4 disulfide bonds was carried out in the following procedures ( 15 ) : 1.5 % of the native TAA in tris buffer of pH 8.6 was reduced with 0.3 M β-mercaptethanol in 8 M urea and 0.01 M EDTA at 40° C for 8 hours. The pH of this reaction mixture was adjusted to and maintained at 8.3 and 1.3 molar excess monoiodoacetic acid to total sulfhydryl groups was added and the solution was left at room temperature for 20 minutes. The solution was dyalyzed against running-tap water and then against deionized water in the dark place. After dialysis for about 48 hours at 5° C, it was lyophlized and stocked. In this sample, 9 moles of S-Carboxymethyl-

cysteine ( S-CM-cysteine ) which corresponds to the total sulfhydryl groups in TAA, were detected by amino acid analysis with an automatic amino acid analyzer ( Beckman Spinco ).

Sonic Oscillation.- - - Oscillator used was " Kubota, type KMS 100 " of 10 KC., 100 W. Fifteen to twenty ml. of the protein solution was subjected to sonic oscillation at 0 - 3° C.

Determination of Bond Splitting.- - - Bond splitting by sonic oscillation was determined by the ordinary ninhydrine colorimetric method ( 16 ) and the newly formed N-terminal amino groups were characterized and estimated by the Sanger's DNFB method ( 17 ).

Viscosity Measurement.- - - Changes of viscosity of protein solution during sonic oscillation were measured by Ostwald type viscometer.

Activity of Enzyme.- - - Amylase activitywas measured by determining its sacharifing power by the method of Fuwa ( 18 ) using amylose as a substrate. Maltosidase activity was measured by determining the quantity of liberated phenol from α-pheryl maltoside of m.p. 190° C, which was prepared according to the method of Matsubara et al. ( 19 ). The $K_m$ and $V_m$

values were estimated from the Lineweaver-Burk's plot ( 20 ).

DEAE-cellulose Chromatography.- - - DEAE-cellulose ( Serva ) was washed with a large volume of 1 N HCl and then with deionized water until the washings became to neutral . Hydrochloride of the cellulose derivative was converted to free base with NaOH and it was washed with large amounts of water to neutral. It was packed to an appropriate glass tube under slight pressure. The column thus prepared was bufferized with the starting buffer and sample solution was placed. The elution was started with the same buffer. The salt concentration was increased gradiently with NaCl by a constant volume mixing chamber of 400 ml.

Gel Filtration.- - - Sephadex G 75 and 100 was washed with large volume of deionized water and fine particles were discarded by decantation. Column of Sephadex was prepared without any pressure.

Amino Terminal Analysis. - - - Amino terminal groups of the sonicated products were determined by the DNFB method ( 17 ). Quantitative estimation of the DNP-amino acids was carried out by two dimensional paper chromatography ( 21 ).

Sedimentation Analysis.- - - Sedimentation measurement was

performed with Hitachi, model UCA-1 ultra centrifuge. The run was carried out at 60,000 r.p.m. and 20° C. Molecular weight of the sonicated product was determined by sedimentation equiribrium method ( 22 ). The run was performed at r.p.m. and 20° C.

Electrophoretic Analysis.- - - Electrophoresis experiments were performed at 12° C by a moving boundary method using Hitachi's electrophoretic Apparatus, Model HT-B.

## EXPERIMENTS AND RESULTS

### The Time Courses of Sonic Oscillation.

In the course of sonic oscillation on the intact TAA at pH 4.2 and R-CM-TAA at pH 7.0, the increment of the number of bond splitting estimated by the ninhydrin colorimetry, the changes of viscosity and the remaining activities are plotted as a function of time as shown in Figs. 1 and 2. In the intact TAA, the increment of the bond creavage at pH 4.2 in $N_2$ gas and in air are 7.0 and 2.5 leucine equivalents per mole of TAA, respectively. In the case of R-CM-TAA at pH 7.0 in air, its number was 4.0 leucine equivalents per mole of R-CM-TAA.

- - - - - - - - - - - - - - - - - - -

Fig. 1. The Time Courses of Sonic Oscillation of the Intact TAA.

- - - - - - - - - - - - - - - - - - -

In grobular state, the intact TAA, the reduced viscosity increased during sonication. While in R-CM-TAA, the linear state, it decreased. Its final values are approximately 7.0 and 5.0, respectively. During sonic treatment of the intact TAA, amylase activity remained nearly constant but maltosidase activity decreased through two steps to about 70 %.

- - - - - - - - - - - - - - - - - -

Fig.2. The Time Course of Sonic Oscillation of R-CM-TAA.

- - - - - - - - - - - - - - - - - -

However an unexpected phenomenon was observed in the case of R-CM-TAA. The once diminished amylase activity, after the reduction of the disulfide groups and subsequent alkylation of the sulfhydryl groups produced, slightly revived and its final specific activity was 1 - 3 % of the intact TAA.

Bond splitting of the intact TAA was measured by two methods, the ninhydrine colorimetry and the DNFB method, as a function of pH. In the case of R-CM-TAA, its bond cleavage was also examined by the two methods at one pH of 7.0, as shown in Fig.3 A. The numbers of the cleaved peptide bonds estimated by the two methods are remarkably different from each other as shown in Figs. 3 A and 3 B.

In the determination of the splitting of peptide bonds due to sonic oscillation by the ninhydrin colorimetry , remarkably different values were observed between sonication experiments in $N_2$ gas and in air at pH 4.2, but such a difference was not observed in the estimation of newly formed N-terminal amino groups by the DNFB method.

- - - - - - - - - - - - - - - - - - - -

Fig. 3. The Time Courses of Peptide Bond Splitting as a Function of pH, Estimated by the Ninhydrin Colorimetry ( A ) and the DNFB Method ( B ).

- - - - - - - - - - - - - - - - - - - -

At any rate, these time curves are of total of newly formed several N-terminal DNP-amino acids, then the ratio of DNP-amino acids estimated to the toral amounts of DNP-derivatives was listed in Table I.

---

Table I. The Ratio of Newly Formed Dnp-amino acids.

---

The difference between the bond splitting numbers estimated by the two methods, might suggest that deamination of $\alpha$-amino group has occurred by the action of sonic oscillation. In order to examine the above possibility, a solution of authentic mixture of several amino acids was subjected to sonic oscillation under the same conditions as for the proteins. The loss of several amino acids by sonic oscillation was observed by the DNP-method. The extent of loss was represented as degradation constant, K ( 1 / min. ) and listed at Table II.

Table II. The Degradation Constants of Amino Acids in Sonic field at Various pH.

The results show that acidic amino acids, aspartic acid and glutamic acid, were considerably destroyed by sonic vibration at a wide range of pH, especially, in alkaline pH, where the other amino acids were also slightly decomposed.

The Products of Sonic Oscillation.

The purification of the sonicated proteins was carried out by DEAE-cellulose column chromatography and by gel filtration. The sonicated TAA ( S-TAA ) for 180 min. at pH 4.2 was neutralized with M/5 $Na_2CO_3$ and dialized against M/25 borate buffer of pH 7.2 for 48 hours. During the dialysis about 25 % of the total $E_{280}$ of the starting solution was lost and the remainning inner solution in the dialysis bag was applied to a DEAE-cellulose column. The chromatogram is shown in Fig. 4.

- - - - - - - - - - - - - - - - - - -

Fig.4 DEAE-cellulose Column Chromatography of S-TAA Obtained by Sonication for 180 min.

- - - - - - - - - - - - - - - - - - -

The main fraction, which was eluted out at the concentration of 0.3 - 0.35 N NaCl, was lyophilized after dialysis against deionized water and was further fractionated by gel filtration ( Sephadex G 75 ). The chromatogram is shown in Fig. 5.

- - - - - - - - - - - - - - - - - - -

Fig. 5. Gel Filtration ( Sephadex G 75 ) of the Main Peak from DEAE-Cellulose Chromatography.

- - - - - - - - - - - - - - - - - - -

Thus obtained two peaks were lyophilized and stocked. They were designated as S-TAA-I and -II, respectively.

These purification procedures are summarized in Table III.

---

Table III. The Purification Procedures of S-TAA II.

---

Characterization of S-TAA-II.

The properties of S-TAA-II, the main product of sonication on the intact TAA, was studied from the points of its physico-chemical, chemical and enzymatic behaiviors.

To compare the chromatographic behavior of S-TAA-II and that of the intact TAA, the mixture of the two proteins was applied to a DEAE-cellulose column. As shown in Fig. 6, the intact TAA and S-TAA-II were eluted from the column at 0.1 - 0.2 and 0.25 - 0.35 N NaCl, respectively.

The chromatographically homogeneous S-TAA-II was analyzed by ultra centrifuge and electrophoraysis, as shown in Figs.

7 and 8, respectively.

- - - - - - - - - - - - - - - - - - -

Fig. 6. Mixed Chromatogram of the Intact TAA and S-TAA-II on a DEAE-cellulose Column.

- - - - - - - - - - - - - - - - - - -

Sedimentation constants of S-TAA-I and -II were calculated to be 4.1 S. and 3.8 S, respectively. Electrophoresis was performed at pH 6.0, 7.0 and 5.0, and in all cases , S-TAA -I and -II behaved as homogeneous materials, respectively.

- - - - - - - - - - - - - - - - - - -

Fig. 7. Sedimentation pattern of S-TAA-II.

- - - - - - - - - - - - - - - - - - -

- - - - - - - - - - - - - - - - - - -

Fig. 8. Electrophoretic pattern of S-TAA-II at pH 5.0.

- - - - - - - - - - - - - - - - - - -

These analyses indicate that S-TAA-II is homogeneous not only chromatographically but also ultracentrifugecally and electrophoretically. To exmine chemical purity of S-TAA -II molecule, the amide terminal group was analyzed. The N-terminal and amide analyses were performed on lyophilized specimen after drying in vacuo to a constant weight. The DNP-protein was hydrolyzed for 8 hours at 105° C. Three kinds of N-terminal amino acids, DNP-Ala, DNP-Glu and DNP-Asp, were obtained. These results are shown in Table IV.

Table IV. The N-terminal DNP-amino acids and amide Content of S-TAA-II.

The molecular weight of S-TAA-II was estimated from the sedimentation equiribrium method as 42,000 ± 2,000. The Archibald pattern is shown in Fig. 9.

- - - - - - - - - - - - - - - - - - -

Fig.9. The Pattern of Archibald Run of S-TAA-II.

- - - - - - - - - - - - - - - - - - -

The amounts of N-terminal amino acids listed in Table IV, were calculated assuming molecular weight of 42,000 for S-TAA-II but the estimated molecular weight from the found N-terminal DNP-Ala is about 35,000.

These results suggest that the followimg two possibilities : ( 1 ) S-TAA-II is contaminated with other sonicated produvts or ( 2 ) has two N-termini and is consisted of at least two polypeptides, which are crosslinked by disulfide linkages. However, judging from the purity and the estimated molecular weight from sedimentation equiribrium method, the former

possibility may be ruled out. Then the latter possibility was examined by reducing the disulfide bridges in S-TAA-II.

The reduction was performed by using two kinds of reducing agents, sodium borohydride and sodium thioglycolate. After the reduction of S-TAA-II with 1 % of sodium borohydride at 25° C, the reaction mixture at zero time and three hours were chromatographed on a DEAE-cellulose column after the blocking the released sulfhydryl groups with PCMB. The chromatographic pattern is shown in Fig. 10.

- - - - - - - - - - - - - - - - - -

Fig. 10. DEAE-cellulose column Chromatography of the Reduced S-TAA-II with $NaBH_4$.

- - - - - - - - - - - - - - - - - -

The breakthrough peak increased as proceeding the reduction. The breakthrough peak was further separated into at least two parts by gel filtration on a Sephadex G 25 column, as

shown in Fig. 11.

- - - - - - - - - - - - - - - - - - - -

Fig. 11. Further Separation of the Reduced S-TAA-II by gel filtration ona Sephadex G 25 Column.

- - - - - - - - - - - - - - - - - - - -

The appearance of sulfhydryl groups in S-TAA-II by 2% $NaBH_4$ at 25° C are plotted in Fig. 12.

- - - - - - - - - - - - - - - - - - - -

Fig. 12. The Time Course of S-TAA-II Reduction with 2 % $NaBH_4$ at 25° C.

- - - - - - - - - - - - - - - - - - - -

The results indicate that the reduction was complete at about one hour and that 5 moles of cystein residue per mole of S-TAA-II. were released. The resulted mixture of 90 min. reduction was also subjected to gel filtration on a Sephadex G 25 column. The elution pattern is shown in Fig. 13.

- - - - - - - - - - - - - - - - - - - -

Fig. 13. Gel Filtration Pattern of Reduced S-TAA-II with $NaBH_4$.

- - - - - - - - - - - - - - - - - - - -

In the case of sodium thioglycolate, the reduction was perform formed at 25° C in 8 M urea of pH 8.6 for 8 hours. The mixture was dialyzed against acetate buffer of pH 6.0 containning $10^{-4}$ M silvernum acetate. The outer solution of dialysis was also applied to gel filtration on a Sephadex G 25 column.

- - - - - - - - - - - - - - - - - - -

Fig. 14. The Pattern of Gel Filtration, on a Sephadex G25 Column, of the outer Solution of Dialysis of the Reduced S-TAA-II with Sodium Thioglycolate.

- - - - - - - - - - - - - - - - -

Finally, several phisicochemical and enzymatic characters of S-TAA-I and -II are listed in Table V, comparing with those of the intact TAA.

---

Fig. 14 The Quantities reflecting the Characters of S-TAA and of the Intact TAA.

---

## DISCUSSION

### The Effects of Sonic Oscillation.

Considering the results of the degradation studies on the effects of sonic oscillation on synthetic linear polymers, it is obvious that its energy is enough to induce chemical changes ( 10, 11 ). Therfore, it is expected that sonic wave modifies the intact TAA molecule, which is known to be relatively resistant to the action of proteolytic enzymes and the thermal agitation ( 8 ).

As shown in Fig. 1, the increment of bond splitting number and the changes of reduced viscosity indicate that the alterations of the primary and tertiary structures of TAA have occurred.

The difference between the numbers of peptide bond split determined by the ninhydrin colorimetry and those by the DNFB method, suggests that deamination of α-amino groups were induced by the action of sonic oscillation in aqueous solution. Previously, it was reported that sonic oscillation has slight oxidative action ( 23 ). But the precise oxidative mechanism was not proposed, because oxidative action was accelerated by the sonication under the stream of innert gas ( 10 ).

At any rate, to check this possibility, the loss of α-amino groups during sonication of the protein under nitrogen atmosphere was compared with that occurred under laboratory atmosphere. ( Fig. 3 ). No remarkable difference between the values estimated by the DNFB method under nitrogen and air. However, when the ninhydrin colorimetry was used, the increment of the ninhydrin values extreamly depressed in the experiments under nitrogen atmosphere. The degree of loss during sonication depends on the natures of side chain groups of amino acid residues and interestingly acidic amino acids are easily destroyed ( Table II ). These results suggest that the treatment of sonic oscillation on protein solution accompany undesirable side reactions, especially deamination. As shown in Table I, the newly formed N.terminal amino acids during sonication of TAA are several and then it leads to the conclusion that the rondom splitting of the protein molecule occurred in the field of sonic wave.

It is clear for the above described facts that sonic treatment are not suitable to isolate a homogeneous active fragment.

The difference of sonic susceptibility on the two polymers, of which comformations are different, groblar and linear, was compared with one onother. The numbers of peptide bond cleaved

were nearly the same in the both cases, TAA and R-CM-TAA ( Figs. 1, 2 and 3 ).

The pH dependence of sonic susceptibility was investigated and obviously pH dependence was observed ( Fig. 3 ). These results might suggest the mechanism of peptide bond splitting by sonic wave but the precise mechanism is unknown in this state.

The reverse phenomena observed in viscosity changes between TAA and R-CM-TAA. These results appear to indicate that the increment of reduced viscosity of the intact TAA may be due to unfolding of the secondary and tertiary structures and that the increment of R-CM-TAA seems to be mainly attributed to destruction of primary structure and partly to reformation of some ordered spatial structure.

TAA maintained nearly constant enzymatic activity during sonic vibration up to 180 min. ( Fig. 1 ). It seems that configurational changes induced by sonication do not affect or have no connection with the structure constituting active center of this enzyme.

While, fantastic results were obtained in the case of R-CM-TAA. It was observed that the once diminished amylase activity by the reduction and following alkylation, revived

by sonic oscillation ( Fig. 2 ). This suggests that sonic wave rearranged the comformation of the inactivated active site contained in R-CM-TAA. For the elucidation of this peculiar phenomenon , further investigations are reqiured and it will be discussed in the later paper in this series ( 24 ).

As a conclusion, it might be said that the action of sonic wave causes alteration of the spatial and primary structures of the protein molecule.

The Product of Sonic Oscillation.

The molecular weight of S-TAA-II estimated from the sedimentation equiribrium method and the quantitative N-terminal amino acid analysis suggest that this derivative of the intact TAA lost a part of molecul of a molecular weight of aproximately 10,000 or a mixture of peptides of which total molecular weight is 10,000 from the original TAA ( Fig. 3 and Table III ).

This chromatographically and electrophoretically homogeneous products has at least two N-termini and cuold be fragmentated into two or three groups by the reduction of disulfide bridges ( Table III. and Figs. 11 - 14 ). These results agree with those of the viscosity studies. The intact TAA has 4 disulfide bridges. The cleavage of the peptide bond

induced by sonication does not accompany remarkable fragmentation without reduction of disulfide linkages and may induce only unfolding or destruction the spatial structure by sonication. Therfore, such unfolding might lead to the increment of the reduced viscosity.

In conclusion, considering the results of purity tests and the estimationmolecular weight by N-terminal analysis and by physicochemical analysis, it seemed that S-TAA-II is consisted of at least two peptide chains , which are liked together with the disulfide bridges and that such comformation may be a rather disordered form than that of the intact TAA. This was also supported by the fact of S-TAA-II was hydrolysed by trypsin under the same conditions, under which the intact TAA was not hydrolysed at all ( 8 ).

## SUMMARY

To degrade the intact TAA molecule, the energy of sonic wave ( 10 KC., 100 W. ) was applied to its aqueous solution.

1.) The effects of sonic oscillation was followed by measuring the changes of viscosity, enzymatic activity and the increment of peptide bond splitting. The bond splitting

number was estimated by the ninhydrin colorimetry and by the DNFB method. The average number of cleavage of peptide bonds under various conditions was about 1 - 7 pre mole of the intact TAA at 180 min. sonication. These were compared with those of R-CM-TAA.

2.) Several kinds of N-terminal amino acids appeared by sonic oscillation. Then, it was conclueded that sonic wave split protein molecule randomly.

3.) The final product of S-TAA-II holds nearly the same enzymatic activity as that of the intact TAA. The molecular weight estimated by the sedimentation equiribrium method was 42,000. But, the molecular weight calculated onthe basis of the estimated N-terminal Ala was 35,000.

Assuming it as 42,000, it was conclueded that S-TAA-II was consisted of at least two peptide chains, of which N-terminal groups were Ala. ( 1 ) and Asp. + Glu. ( 1 ), and that the two peptide chains are crosslinked by disulfide bonds with each other. The above considerations were supported by the fragmentation studies with reduction of the disulfide bridges.

4.) R-CM-TAA, which seems to have no definite secondary and tertiary structures, was also split by sonication with

nearly same extent as the intact TAA, accompaning decrement of viscosity and the reappearance of the once diminished amylase activity.

## ACKNOWLEDGEMENTS

The author wish to express his thanks to prof. Narita , prof. Takashima and Dr. Toda for their kind guidances and valuable discussions through this investigation, to Dr. Kakiuchi for his help for sedimentation studies and also to Sankyo Co., Ltd. for their kind supply of " Taka-diastase Sankyo ".

## REFERENCES

1.) Nylander, O., and Malmström, B.G., Biochim. et Biophys. Acta., 34, 196 ( 1959 )

2.) Viswanatha, T., and Liner, I. E., Biochim. et Biophys. Acta, 37, 389 ( 1960 )

3.) Funatsu, N., and Tokuyasu,K., J. Biochem., 46, 1441 ( 1959 )

4.) Hill, R.L., and Smith, E. L.,J. Biol. Chem., 235,2332 ( 1960 )

5.) Toda, H., and Akabori, S., J. Biochem., 53, 95 ( 1963 )

6.) Toda, H., J. Biochem., 53, 425 ( 1963 )

7.) Toda, H., J. Biochem., 54, 1 ( 1963 )

8.) Okunuki, K., Hagihara, B., Matsubara, H., and Nakayama, T., J. Biochem., 43, 453 ( 19 56 )

9. ) Ikenaka, T., J. Biochem., 46, 297 ( 1959 )

10.) Grabar, P., Advances in Biological and Chemical Physics, 3, 191 - 237 ( 1960 ) Academic Press.

11.) Schmid, von G., " Der Ultraschall andder Medizin " , 81 ( 1949 )

12.) Akabori, S., Hagihara, B., and Ikenaka, T., J. Biochem., 41, 577 ( 1954 )

13.) Toda, H., and Akabori, S., J. Biochem., 53, ( 1963 )

14.) Narita, K.,Murakami, H., and Ikenaka, T., will be published.

15.) Narita, K.,Toda, H., and Seon, B., will be published.

16.) Moore, S., and Stein, W. H., J. Biol. Chem., 211, 907 ( 1954 )

17.) Sanger, F., Biochem. J., 39, 508 ( 1945 )

18.) Fuwa, H., J. Biochem., 41, 583 ( 1954 )

19.) Matsubara, S., Ikenaka, T., and Akabori, S., J. Biochem., 46, 425 ( 1959 )

20.) Lineweaver, H., and Burk, D., J.Am.Chem. Soc., 56, 658 ( 1934 )

21.) Frankel-Conrat, H., Harris, J.I., and Levy, A.L., in D. Glick, " Methods of Biochemical analysis, Vol. II, Interscience Publishers, Inc., New York, 1995, p. 359.

22.) Archibald, W. J., J. Phys. Chem., 51, 1204 ( 1947 )

23.) Richards, W.T., and Loomis, A. L., J. Am. Chem. Soc., 49, 3086 ( 1927 )

24.) The third and forth reports of this sereis.

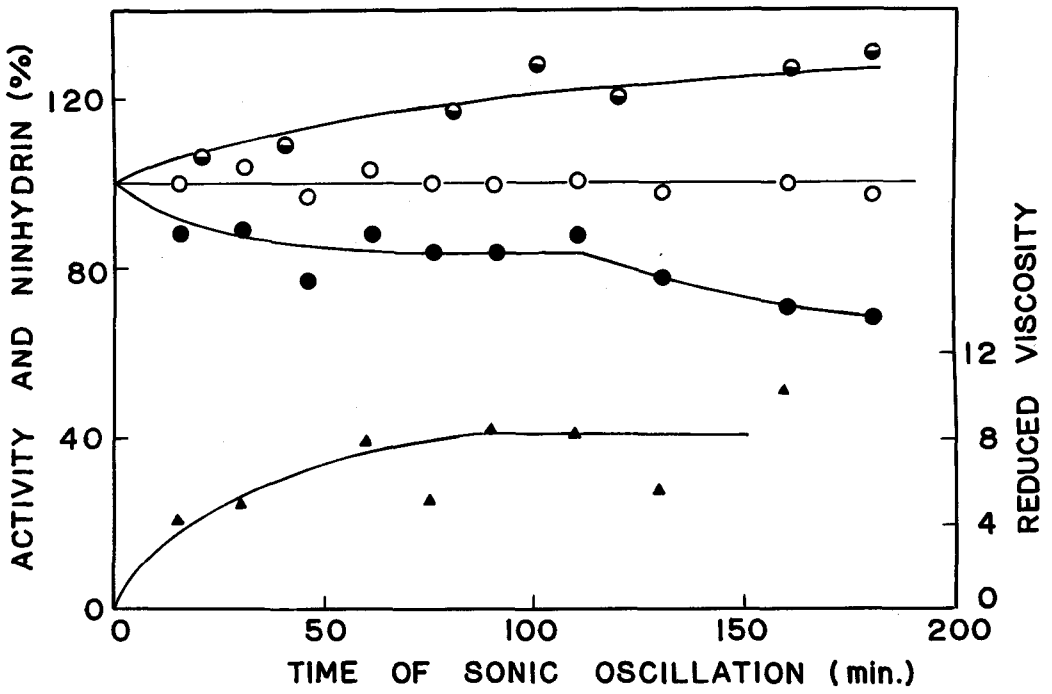



Fig. 1, Time Curves of Sonic Oscillation on the Intact TAA. The Conditions of sonication. ; 15 ml. of 1 % of pH 4.2 at 0° - 3° C. —●—, Ninhydrine ; —○—, Amylase Activity ; —●—, Maltosidase Activity ; —▲—, Reduced Viscosity.

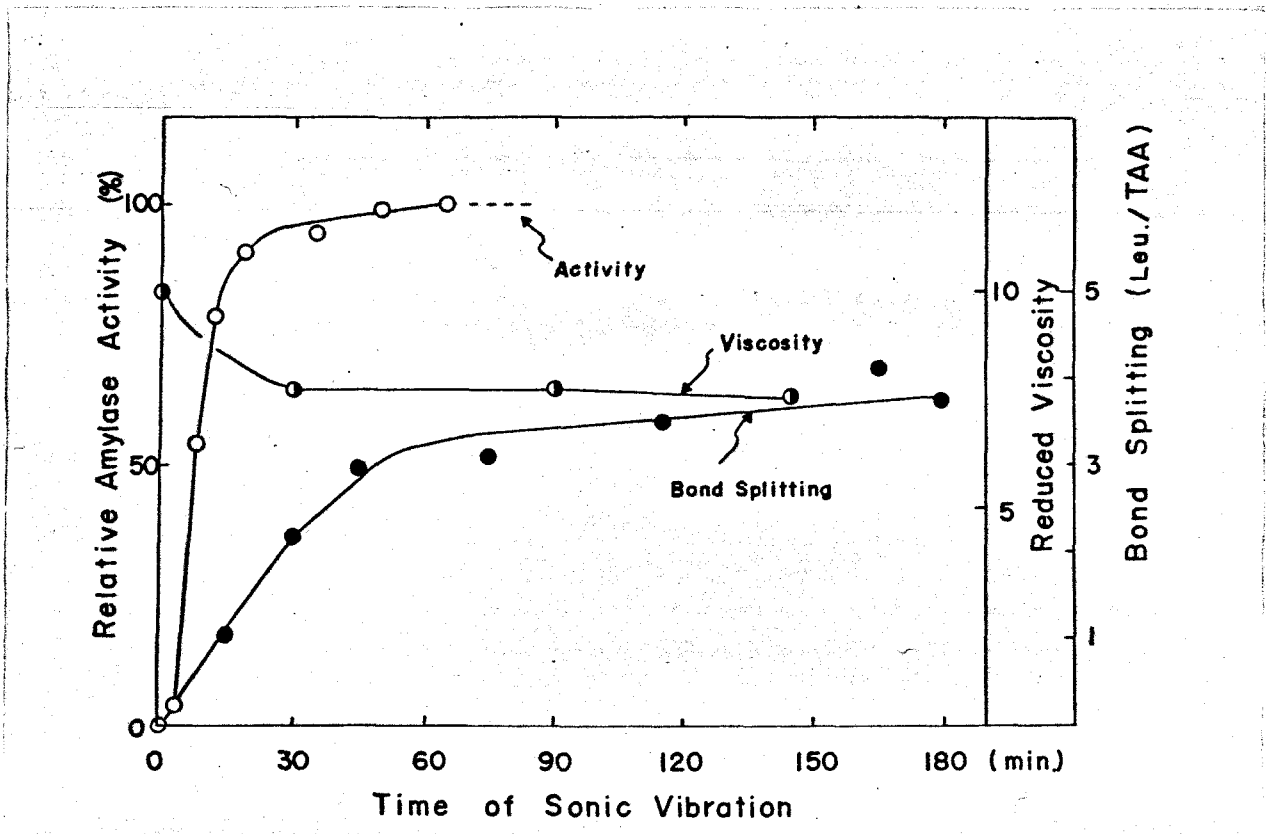



Fig. 2, Time Curves of Sonic Oscillation of R-CM-TAA.

The conditions of the sonication ; 15 ml. of 0.5% aqueous solution of R-CM-TAA containing $10^{-3}$ M $Ca^{++}$.

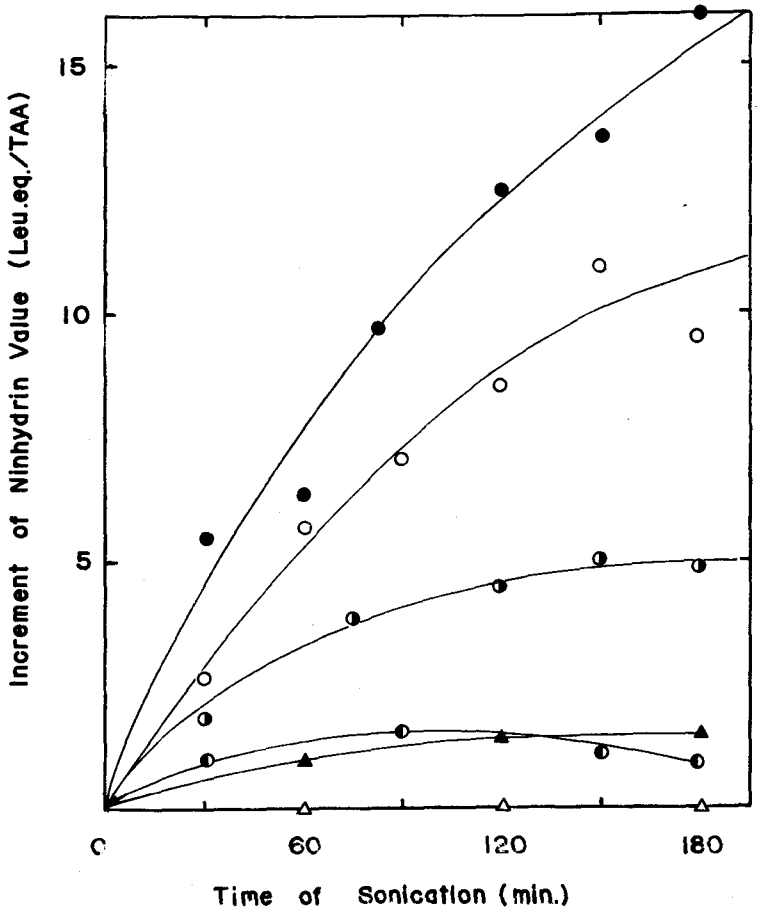



Fig. 3. ( A ). Increment of Ninhydrin Values Induced by Sonication under Several Conditions.

—●—, pH 7.2 ( $N_2$ ) ; —◑—, pH 4.2 ( $N_2$ ) ; —◑—, pH 9.0 ( $N_2$ ) ; —△—, pH 2.0 ; —▲—, pH 4.2 ( air ) ; —○—, R-CM-TAA at pH (7.0 ( $N_2$ ).

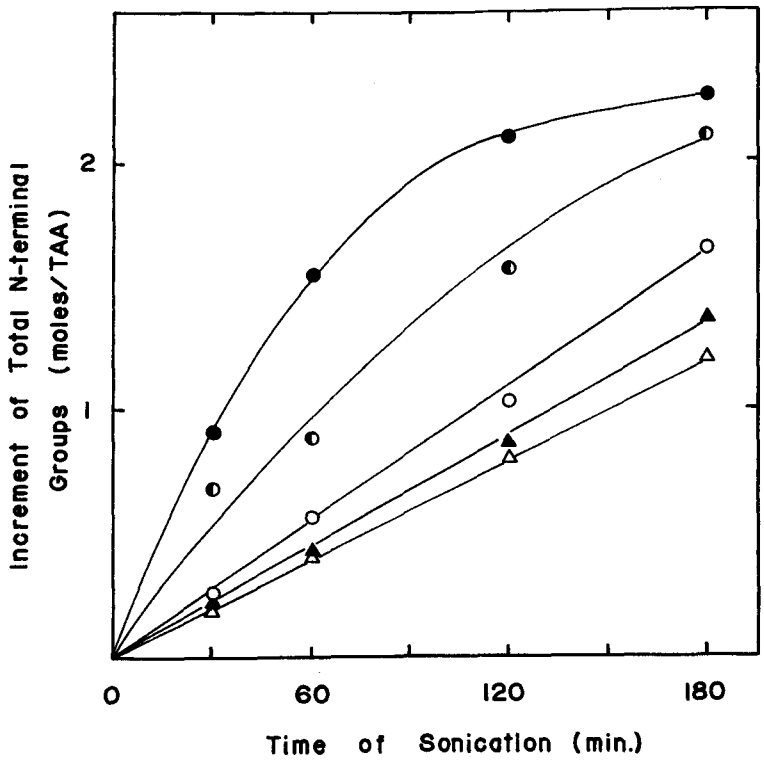



Fig. 3. ( B ) : Increment of N-terminal DNP-amino Acids.

—●—, pH 4.2 ( Air ) ; —◐—, pH 4.2 ( $N_2$ ) ;

—▲—, pH 7.2 ( $N_2$ ) ; —△—, pH 9.0 ( $N_2$ ) ;

--○--, R-CM-TAA at pH 7.2 ( $N_2$ ).

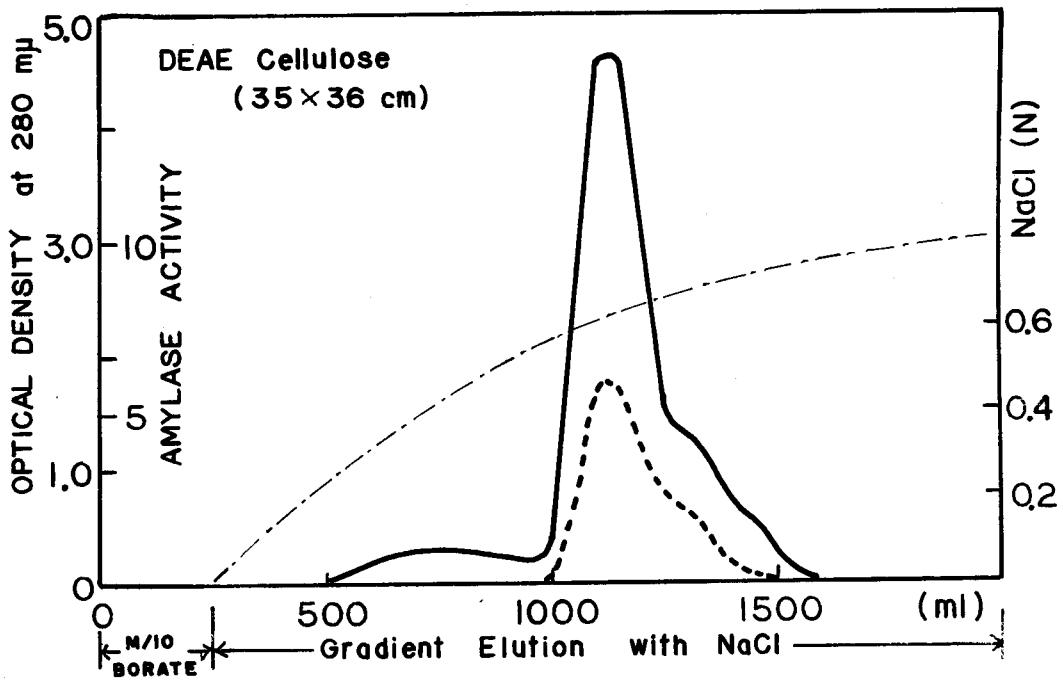



Fig. 4. Chromatographic Pattern of Sonicated TAA on a DEAE-Cellulose Column ( 3.5 × 36 cm. ).

———, Optical Density at 280 mu ; ----, Amylase Activity at 500 mu.

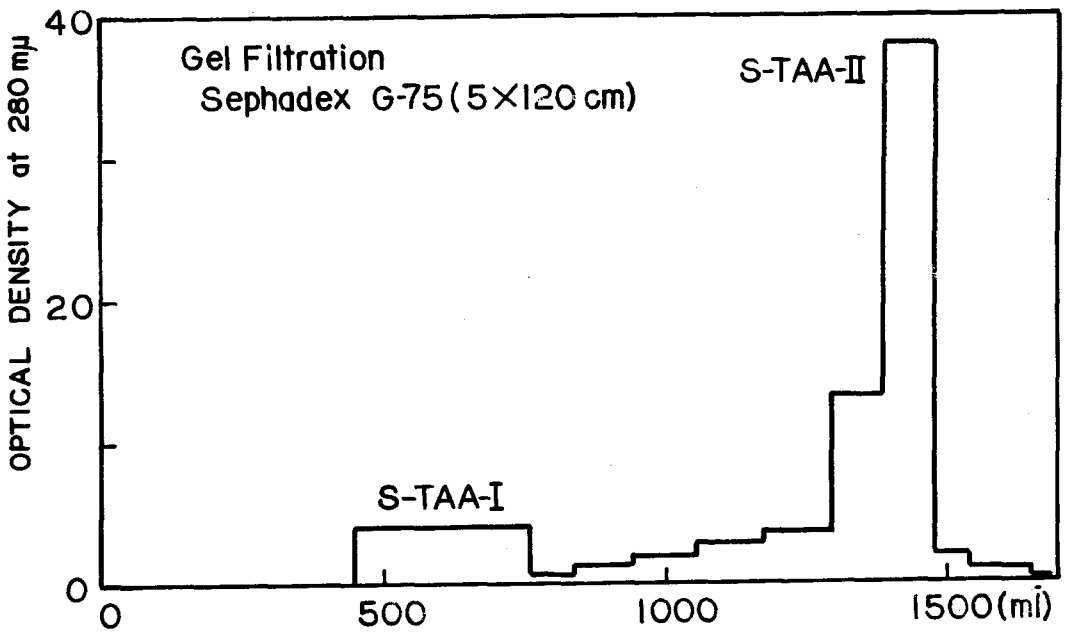



Fig. 5. Pattern of Gel Filtration of Chromatographycally Purified Sonicated TAA on a Sephadex G 75 Column ( 5 X 120 cm. ). Deionized water was used for eluent.

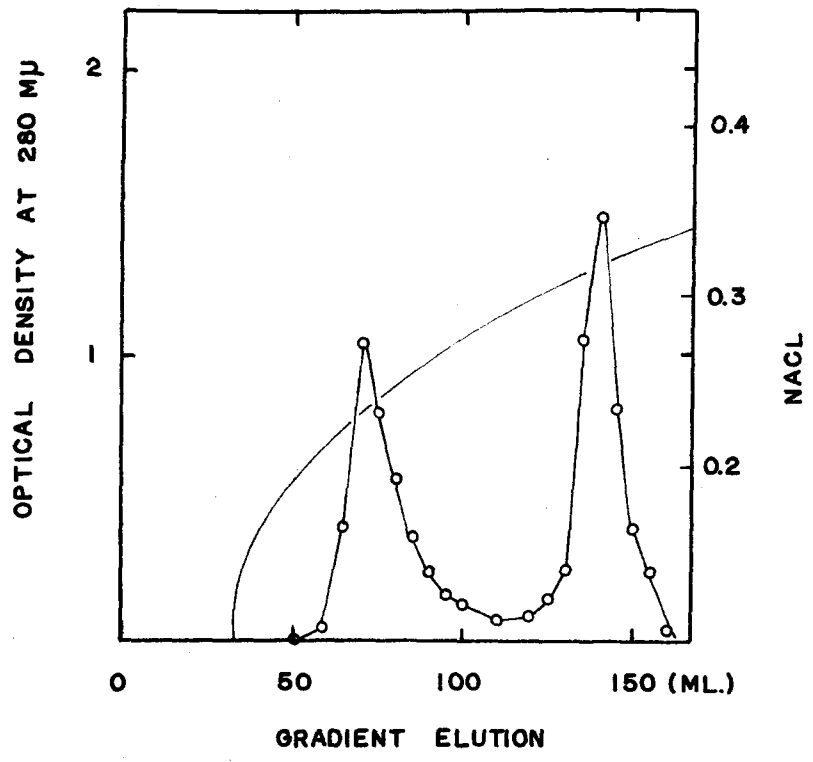



Fig. 6. Mixed Chromatography of the Intact TAA and S-TAA-II on a DEAE-CEllulose Column.

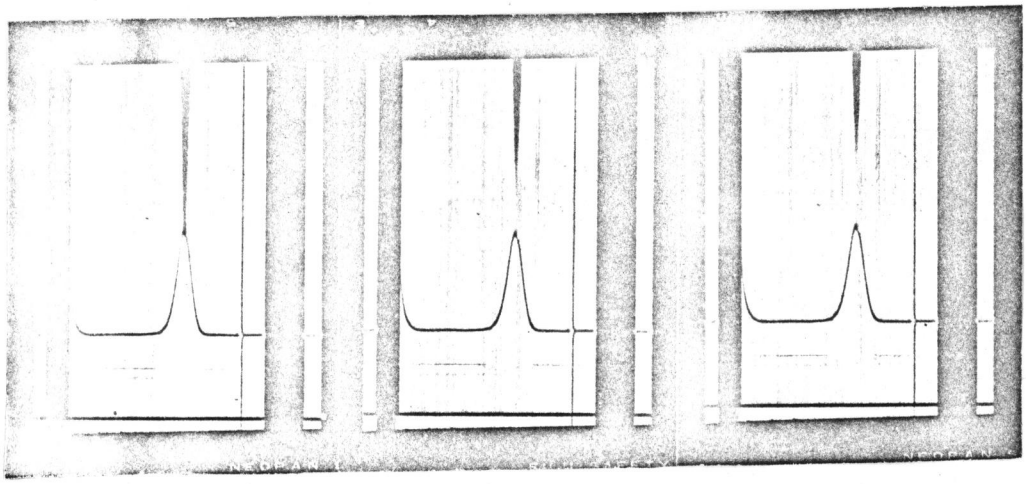


Fig. 7. Sedimentation Pattern of S-TAA-II.

1 % and $\mu = 0.1$.

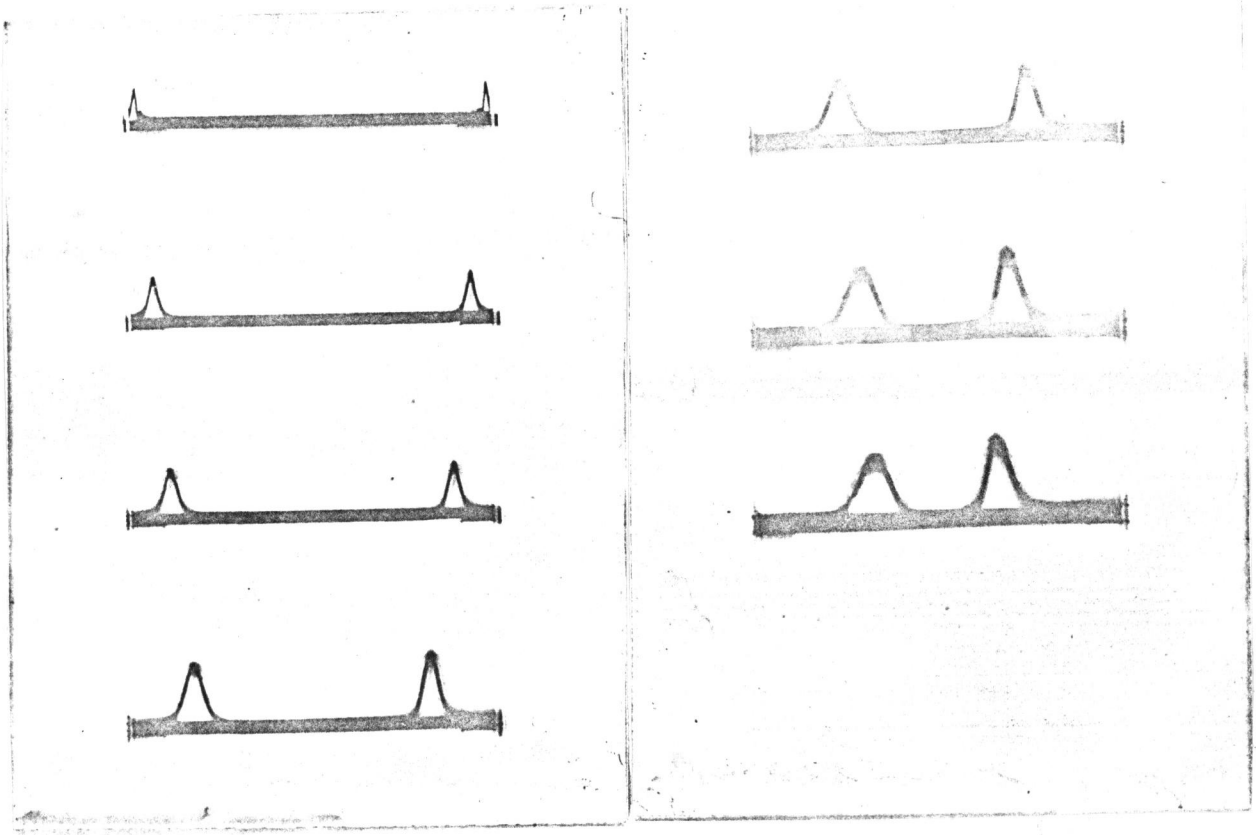


Fig. 8. Electrophoretic Pattern of S-TAA-II at pH 5.0, 12° C, and $\mu$ = 0.1.

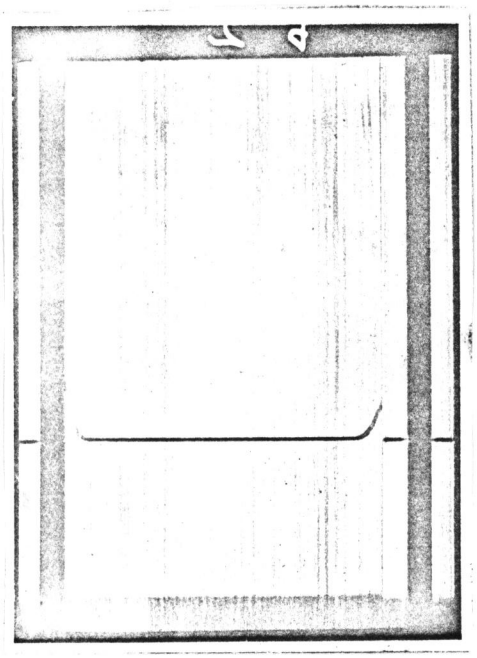


Fig. 9. The Pattern of Archibald Run of S-TAA-II.

$\mu = 0.1$.

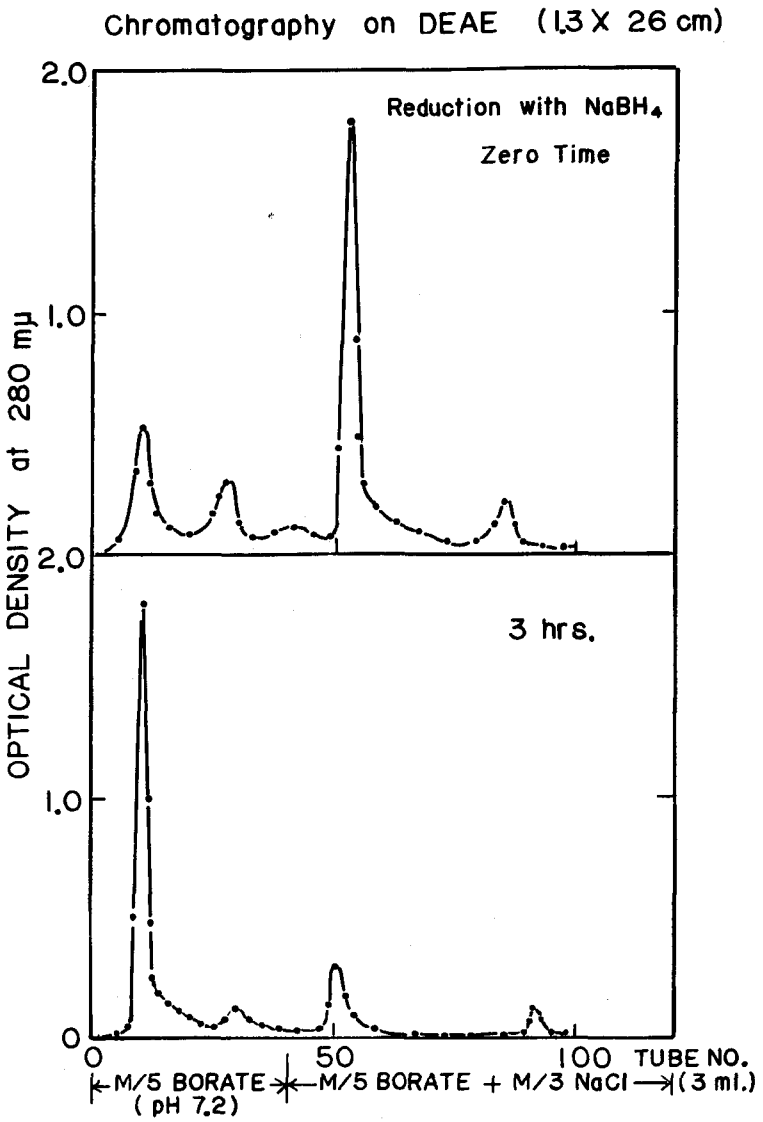



Fig. 10. Chromatogram of the Reduced S-TAA-II with $NaBH_4$, on a DEAE-Cellulose Column ( 1.3 X 26 cm. ).

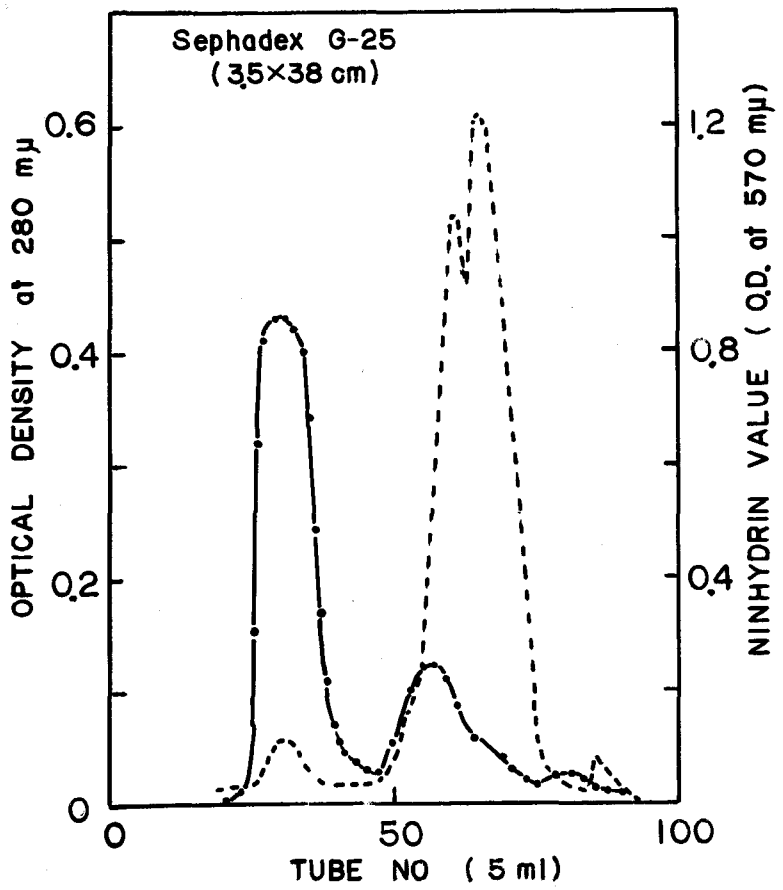



Fig. 11. Chromatogram of the Reduced S-TAA-II which was purified by DEAE-cellulose Chromatography ( Fig. 10 ), on a Sephadex G 25 Column (( 3.5 × 38 cm. ).
——, Optical Density at 280 mu ; ----, Optical Density at 570 mu.

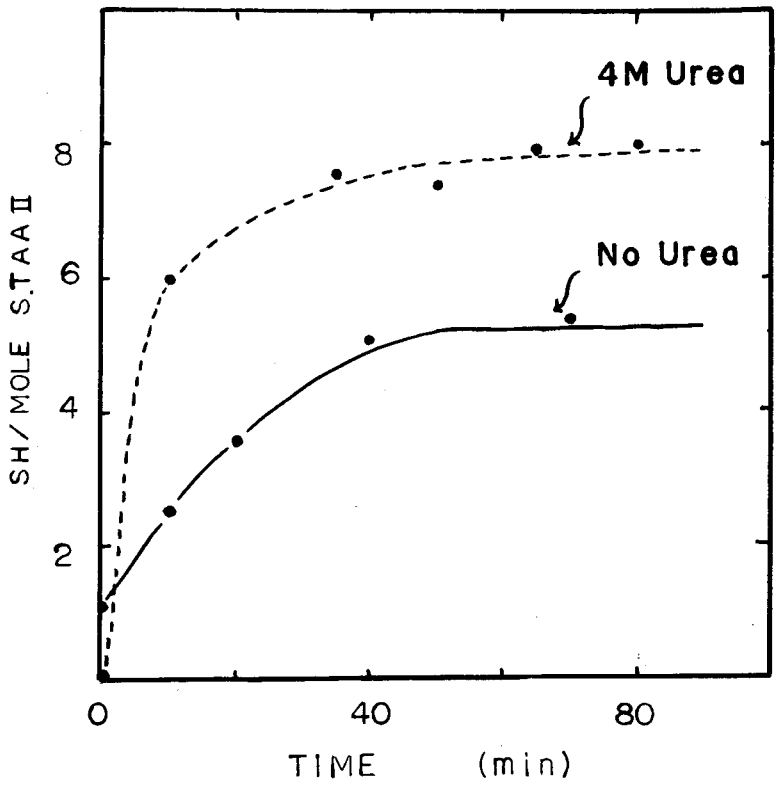



Fig. 12. Reduction Time Curve of S-TAA-II.

1 % of S-TAA-II and 2 % of $NaBH_4$ at 25° C.

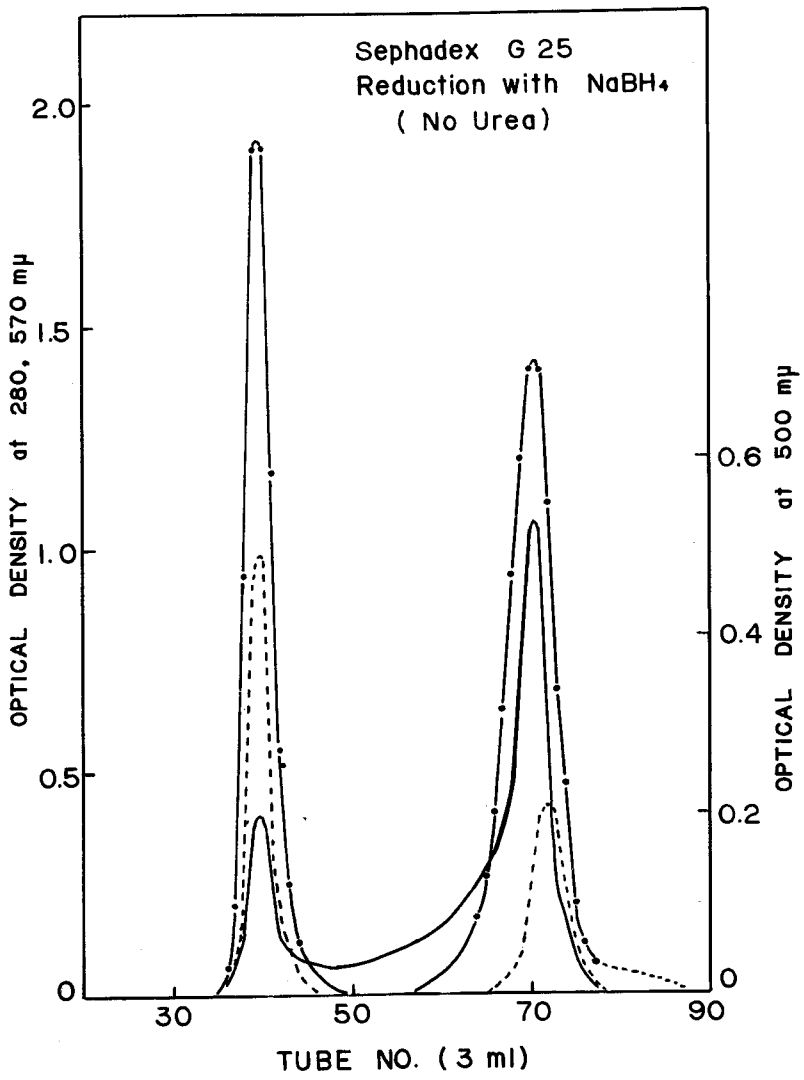



Fig. 13. Chromatographic Pattern of the Reduced S-TAA-II on a Sephadex G 25 Column ( 4 × 90 cm. ).

—●—, Optical Density at 280 mu ; ——, Optical Density at 570 mu ; - - -, Amylase Activity at Optical Density at 500 mu.

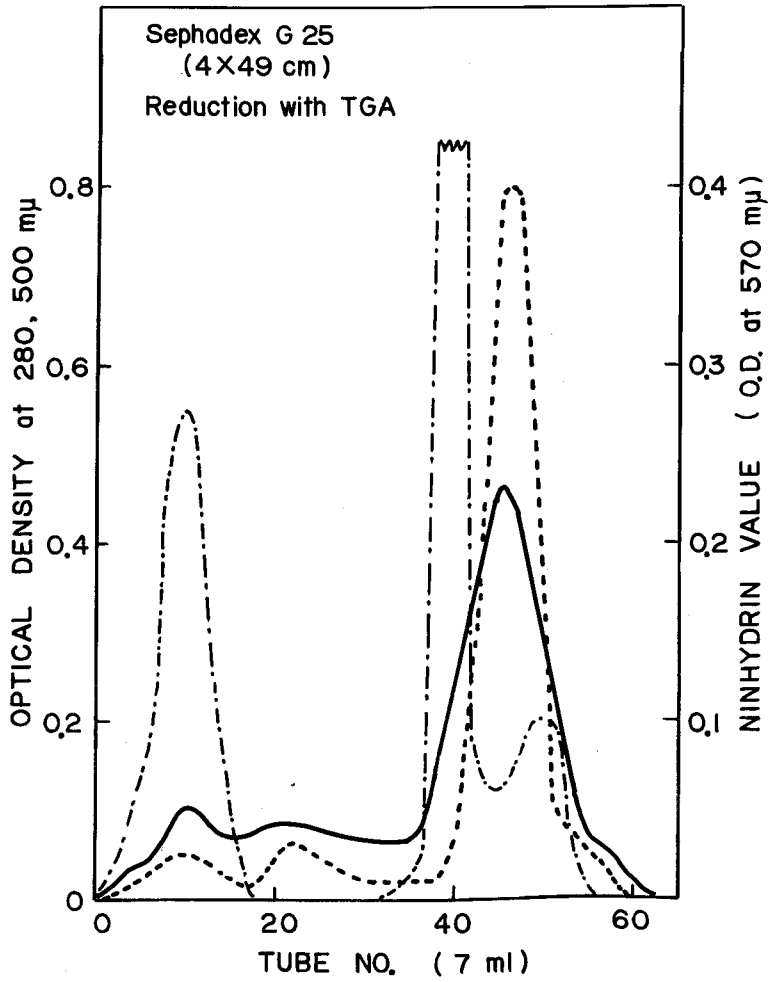



Fig. 14. Chromatographic Pattern of the Reduced S-TAA-II with Sodium Thioglycolate, On a Sephadex G 25 Column ( 4 X 49 cm. ).

Table I.

The Ratio of Newly Formed N-terminal Amino Acids by Sonication of the Intact TAA at Several PH's.

| Amino Acids | The Ratio of Newly Formed N-terminal Amino Acids ( % ) | | | | |
|---|---|---|---|---|---|
| | pH 7.0* ( $N_2$) | pH 4.2 ( N2) | pH 4.2 ( air ) | pH 7.0 ( $N_2$) | pH 9.0 ( $N_2$) |
| Glu. | 6.9 | 13.9 | 11.2 | 5.2 | 16.1 |
| Asp. | 17.3 | 18.3 | 21.2 | 9.9 | 32.1 |
| Ala. | 0.1 | 15.1 | 5.2 | 10.6 | 7.3 |
| Gly. | 16.7 | 13.1 | 17.5 | 6.9 | 18.1 |
| Thr. | 2.6 | 8.3 | 17.4 | 11.3 | 10.2 |
| Ser. | 22.4 | 2.6 | 9.3 | 28.0 | 16.1 |
| Leu./Val. | 3.2 | 14.7 | 5.2 | 12.2 | —— |
| Lys. | —— | 14.1 | —— | 15.6 | —— |

* The values of R-CM-TAA.

Table II.

The Degradation Constant of Amino Acids in Sonic Field at Various PH's.

| PH | Degradation Constant for Several Amino Acids ( $1/min. \times 10^2$ ) | | | | | |
|---|---|---|---|---|---|---|
| | Ala. | Gly. | Ser. | Thr. | Asp. | Glu. |
| 4.2 ( $N_2$ ) | 0.0 | 0.8 | 0.0 | 0.4 | 2.0 | 2.6 |
| 4.2 ( air ) | o.0 | 0.0 | 0.0 | 0.4 | 2.5 | 2.2 |
| 7.0 ( $N_2$ ) | 0.2 | 0.0 | 0.5 | 0.8 | 0.7 | 0.2 |
| 9.0 ( 0.5 | 0.5 | 0.0 | 1.4 | 1.2 | 2.2 | 2.5 |

These value were obtained from the results of 10 min.' sonication for a mixture of the authentic mixtureacids under the same conditions as for the protein solution.

Table III.

Preparation Schedule of Sonicated TAA.

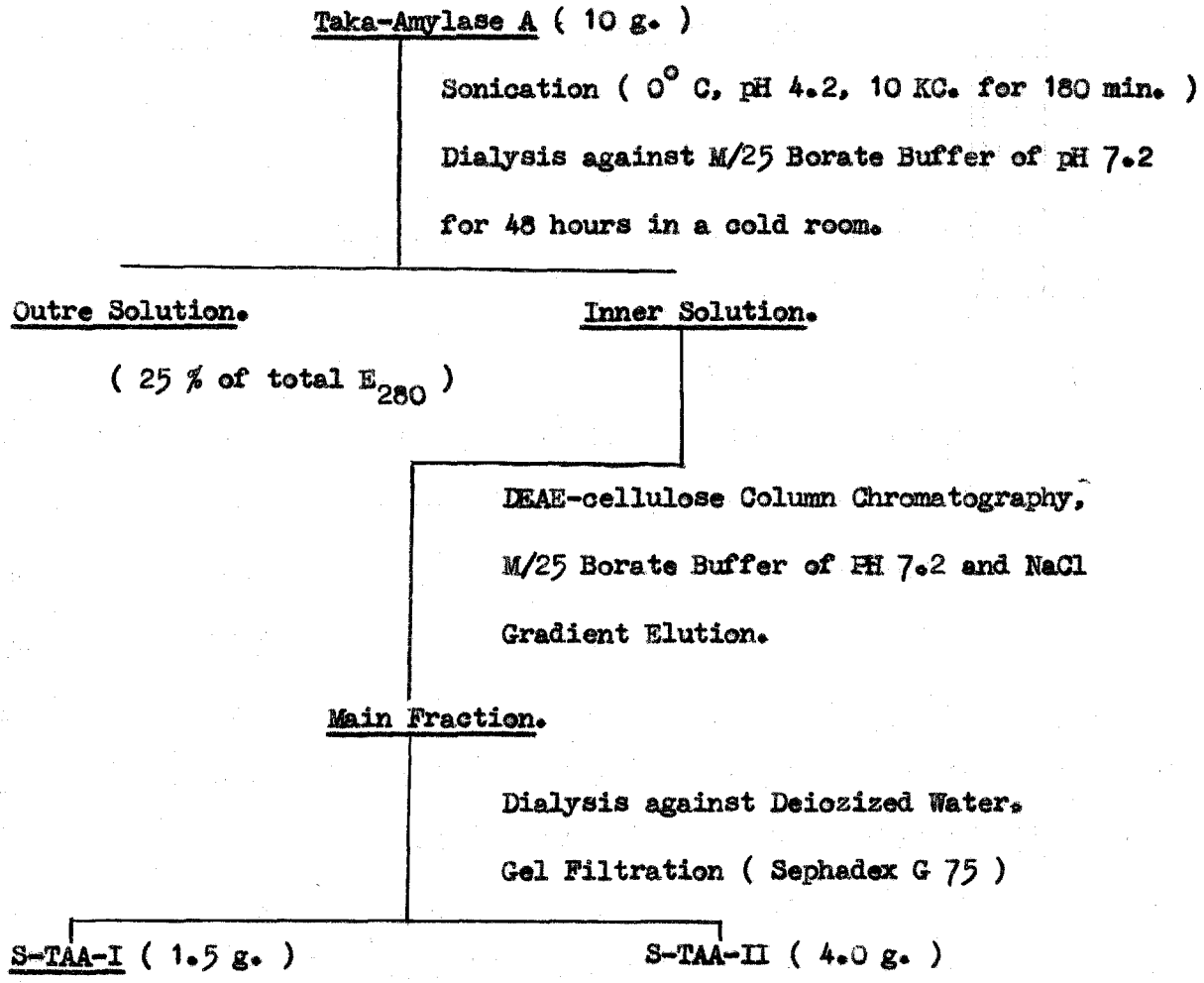


Table IV.

N-Terminal Group Analysis of S-TAA-II and Its Amide Content.

| Proteins. | Moles of DNP Amino Acid per Mole of Protein. | | | Amide Content ( % ) |
|---|---|---|---|---|
| | DNP-Ala. | DNP-Asp. | DNP-Glu. | |
| TAA | 0.97 | ——— | ——— | |
| S-TAA-II | 1.14 | 0.76 | 0.16 | 1.49 |

Molecular weight of the intact TAA and of S-TAA-II were assumed as 51,000 and 42,000, respectively.

Table V.

Several Properties of S-TAA-II and Their Comparison with of TAA.

| | TAA | S-TAA-I | S-TAA-II |
|---|---|---|---|
| Molecular Weight | 53,000 | ——— | 42,000 |
| $S_w$ | 4.1 S | 4.4 S | 3.8 S |
| Intrinsic Viscosity | 3.3 | 8.5 | 4.5 |
| Nitrogen Content ( % ) | 15.3 | 14.1 | 15.3 |
| Extinction Coeff. | $11.7 \times 10^7$ | ——— | $8.6 \times 10^7$ |
| Activity / mole | 1.0 | ——— | 0.7 |
| Activity / N. | 1.0 | 0.9 | 0.9 |
| $K_m$ ( amylose ) | $3.0 \times 10^{-5}$ M | ——— | $1.6 \times 10^{-5}$ M |
| $K_3$ ( amylose ) | $1.2 \times 10^3$ /sec. | | $2.6 \times 10^2$/sec. |

Molecular weight of the intact TAA and of S-TAA-II were assumed as 51,000 and 42,000, respectively.

The measurement of intrinsic viscosity was performed at 20° C by using M/5 borate buffer of pH 7.2 and extinction coefficient at 280 mu was measured in deionized water.

Effects of Sonic Oscillation on Taka-Amylase A and Its Derivatives.

II. On the Crystallization of Sonicated Taka-Amylase A and Its Some Characters.

By Ikunoshin Kato

# Effects of Sonic Oscillation on Taka-Amylase A And Its Derivatives.

## II. On the Crystallization of Sonicated Taka-Amylase A And Its Some Characters.

In the preceeding paper, it was reported that the intact TAA was degraded by sonic oscillation of 10 KC., 100 W. and the main product ( S-TAA-II ) was isolated by chromatography on a DEAE-cellulose column and gel filtration on a Sephadex G 75 column. The active derivative thus obtained has molecular weight of about 42,000 by the sedimentation equiribrium method and was shown to be homogeneous chromatographically and electrophoretically.( 1 )

However, judging from the N-terminal analysis ( 1 ), S-TAA-II has three kinds of N-terminal amino acids and this active derivative supposed to be still not homogeneous. Therefore, its crystallization was attempted for further purification.

The present paper represents its crystallization procedures, some characters of the resulted crystalline S-TAA-II and its amino acid composition, comparing with those of the intact TAA.

## METHODS AND MATERIALS.

Crystallization of S-TAA-II.- - - All procedures were performed at 3 - 5° C.

Electrophoresis. - - - Electrophoresis was performed by using an electrophoretic apparatus of Hitachi model HTB. at 20° C.

N- terminal Analysis.- - - Amino terminal groups were determined quantitatively bt the method of DNFB ( 2 and 3 )

Amino Acids Analysis.- - - Hydrolysis of protein was carried out in the following procedures ; 10 mg. of the crystalline S-TAA-II was hydrolyzed with twice distilled HCl in a sealed evacuated tube for 24 hours at 100° C. After removal of the excess HCl under reduced pressure on an evaporator at 50° C, the hydrolysate was transfered quantitatively into a volumetric flask with 0.2 N citrate buffer of pH 2.2. Aliquates were applied to the column of Amberlite IR -120, 150 × 0.9 cm. and 15 × 0.9 cm. of Beckman Spinco Amino Acid Analyser, respectively

Tryptophane was determined according to the spectrophotometric method of Goodwin and Morton ( 4 )

Cystine and cystein were determened by amperometric titration according to Benesh et al. ( 5 ) after reduction

of the protein with $NaBH_4$.

Archibald Run.- - - This was performed by using an analytical ultracentrifuge of Hitachi Model E equipped Schlieren Optics. The run was carried out at 15,590 r.p.m. and 20° C.

The molcular weight was calculated from the following equation ( 6 ) ;

$$M = RT / ( 1 - v )^2 \dot{r}c \; dc / dr$$

The density of the solution ( v ) and of the solvent were measured at 20° C using the pycnometer of Ostwald type of the capacity of about 1 ml.

Enzymatic Activity.- - - Amylase activity was assayed by estimating its sacharifing power for amylose ( Nagase ) in M/ 5 acetate buffer of pH 5.3 according to the method of Fuwa ( 7 ).

## EXPERIMENTS AND RESULTS.

Crystallization of S-TAA-II.- - - One per cent solution of S-TAA-II in M3/ 40 calcium acetate of pH 6.5 was kept at 2 - 3° C in an ice bath and cold acetone was slowly added to the solution with constant stiring to the final concentration of about 20 %. The resulted cloudy solution was left over night in a cold room at 3 -5° C, then it was centrifuged. The supernatant was again allowed to stand for 1 - 3 days in a cold room by addeng cold acetone to the concentration of 30 % ( V/V ) of the original protein solution.

By this procedures, crystallization proceeded and the yield was 50 - 60 %. The crystalline form is shown in Fig. 1.

- - - - - - - - - - - - - - - - - - -

Fig. 1. Photograph of the Crystalline S-TAA-II.

- - - - - - - - - - - - - - - - - - -

As shown in Fig. 1, the form of crystal was similar to

Table I. The N-terminal Analysis of Crystalline S-TAA-II

The results indicate that the crystalline S-TAA-II was a sole N-terminal alanine and N-terminal aspartic acid and glutamic acid which were found in the crude S-TAA-II ( 1 ) disappeared. The molecular weight of crystalline S-TAA-II based on the N-terminal alanine was estimated to be 36,000 ± 2,000.

Archibald Run.

Previously, the molecular weight of the amorphous S-TAA-II was estimated as 42,000 from the sedimentation equiribrium method ( 6 ). Then it was again measured on the crystalline S-TAA-II. At the present time, it was estimated to be 37,000 ± 1,000. The pattern was shown in Fig. 3.

- - - - - - - - - - - - - - - - - - - -

Fig. The Pattern of Archibald Run of Crystelline S-TAA-II

- - - - - - - - - - - - - - - - - - -

Amino Acid Composition of Crystalline S-TAA-II.

Judging from the results of electrophoretic and N-terminal analyses, the crystalline S-TAA-II seems to be uniform. Then its amino acid analysis was performed and the results were compared with those of the intact TAA ( 9 ).

- - - - - - - - - - - - - - - - - -

Table II. Amino Acid Composition of the Crystalline S-TAA-II

- - - - - - - - - - - - - - - - - - -

The molecular weight estimated from the physicochemical measurement, 37,000, was used to calculate the number of

amino acid residues present in the enzyme. These data are presented in Table II. With use of the average of the molecular weights calculated from each amino acids, a Value 36,918 was obtained for the mean molecular weight of crytalline S-TAA-II.

In Table III, tentative classification of side chains of amono acid residues which were containd in the enzyme is presented. Comparing it with that of the intact TAA ( 9 ), no remarkable difference is observed.

---

Table III. Calculation of Side Chains in the Crystalline S-TAA-II and their Comparison with those of the Intact TAA.

---

Enzymatic Properties for Amylose.

The enzymatic character of crystalline S-TAA-II was

examined comparing with that of the intact TAA. The kinetic constants of the enzyme fragment and the intact TAA are listed in Table IV.

---

Table IV. Kinetic Constants of Crystalline S-TAA-II and the Intact TAA.

---

These data show that the molar specific amylase activity of the enzyme was 60 % of the original TAA. However, such overall rate does not indicate the precise enzymatic character, and $K_m$ and $V_m$ were obtained from the Lineweaber-Burk's plot ( 10 ) as represented in Table IV. The value of $K_m$ of S-TAA-II was the same order with that of the intact TAA, but $V_m$ remarkable differed from of TAA. Elucidating further evident character of S-TAA-II, temperature effect on the rate was studied and thermodynamic constants were calculated.

In Fig. 4, temperature effect on the rate for amylose was shown.

- - - - - - - - - - - - - - - - - - - - -

Fig. 4. The Plott of Log K/T versus 1/T of the Crystalline S-TAA-II and the Intact TAA for Amylose.

- - - - - - - - - - - - - - - - - - - -

The thermodynamic constants ewere calculated from the data in Fig. 4. According to the theory of " Absolute rate process " ( 11 ), the values were estimated as shown in Table V. Comparing the values of H and S for amylose of S-TAA-II with those of the intact TAA, it is evident that bothe values of the former are a little higher than those of the latter.

Trypsin Susceptibility of Crystalline S-TAA-II.

To examine the molecular weight of the crystalline S-TAA-II, trypsin susceptibility was studied at pH 7.4 , 8.1 and 8.9 at 20° C. The time curves followed by the

Table V. Thermodynamic Quantities of Crystalline S-TAA-II and the Intact TAA for Amylose.

ninhydrin colorimetry were represented in Fig. 5. Trypsin used was 5 % ( W/W ) of the crystalline S-TAA-II.

The changes of amylase activity, was also followed but no detectable loss was observed at the three pH's.

Fig. 5. The Time Curves of Tryptic Digestion of Crystalline S-TAA-II at three pH's.

The resulted product at 5 hours' tryptic digestion at pH 8.1 was applied to gel filtration on a Sephadex G 100 column and its pattern is shown in Fig. VI.

- - - - - - - - - - - - - - - - - - - -

Fig. VI. Gel Filtration of Tryptic Digestion product of Crystalline S-TAA-II.

- - - - - - - - - - - - - - - - - - -

## DISCUSSION.

### Crystallization.

As described before, an attempt to crystallize S-TAA-II was successful. Therfore it is evident that sonic treatment has not greatly changed the spatial configulation of the intact TAA and that S-TAA-II contains partly rather rigid structure. Toda et al. ( 12 - 14 ) have also obtained a crystalline active derivative of TAA, of which molecular

weight was 35,000, by proteolytic digestion. Considering their data in combination with our results, it is supposed that TAA has a structure having a highly regenerating potency or a strikingly stable conformation and stable active center.

By the crystallization of amorphous S-TAA-II, its heterogenity on the N-terminal amino acids diminished ( Table I. ). The above results lead to the conclusion that N-terminal glutamic acid and aspartic acid, which were formed in the amorphous S-TAA-II, are derived from the miner components and that the S-TAA-II became crystalline by removal of the contaminated minor components. ( 1 ).

Amino Acid Composition.

The data of amino acid composition ( Table II. and III. ) indicate that the peptides consisting of about 120 residues are releases from the intact TAA by sonic action leading to the S-TAA-II, of which amino acid composition is similar to that of the intact TAA.

Molecular weight of Crystalline S-TAA-II.

The molecular weight of crystalline S-TAA-II based on the amount of the N-terminal alanine and on the results of the physicochemical method are essentially agreement with the calculated molecular weight for each amino acid from

the most probable integer ( Tables I - III ).

Enzymatic Character.

Judging from the overall rate for amylose ( Table IV ), the enzymatic activity of the modified enzyme are aproximately the same as that of the original TAA and loss of about 120 amino acid residues did not change appaerently the enzymatic activity. However, comparing the thermodynamic quantities of the S-TAA-II with that of the intact TAA, the catalytic efficiency are considerably decreased ( Table V ). Such changes induced by the present modification are similar to those for pepsin reported by Funatsu ( 15 ) and Toda ( 13 ).

According to Eyring ( 11 ), a reaction rate depends on two quantities, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. When compared with of the intact TAA, the increment of both quantities was induced by the modification. Therfore, the overall enzymatic activity remains as nearly constant owing to the balance of the two quantities, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. These quantities were calculated from the overall rate , not from elementary rate constant. So, what part of rate prosecc contribute to thermodynamic quantities obtained could not be understood.

When $K_m$ and $V_m$ values were considered, $K_m$ was not changed and $V_m$ obviously decreased by this modification. It indicates

that $K_1$ and $K_3$ were decreased with the same proportion, if an assumption of $K_m = K_3/K_1$ was made.

Tryptic Susceptibility.

The intact TAA was not hydrolysed by trypsin ( 16 ), but the present modified enzyme is succeptible as presented in Fig. 5. This phenomenon suggests that the crystalline S-TAA-II have a partly loosed structure.

Effects of Sonic Oscillation.

Grabar and Kaminsky ( 17 ) have observed that even if chemical changes, the loss of $E_{280}$ and its fragmentation, were induced by ultrasonic oscillation, the antigenecy of egg alubmine was not lost.

As descrived, in the case of TAA, it holds nearly the same enzymatic activity. Considering these results, it might be concluded that sonic wave has a limitted destructing function of a molecule.

As a conclusion, it seems that the finally purified product of the crystalline S-TAA-II is a uniform molecule, which was produced from the intact TAA missing peptides of molecular weight of 10,000 - 15,000. Such modification does not induce the alteration of an essential structure and the enzymatic character of the original TAA.

## SUMMARY

1.) S-TAA-II, wich is a sonicated main product derived from the intact TAA, was crystallized from aqueous acetone. The crystalline S-TAA-II was examined on its purity by moving boundary electrophoresis and N-terminal analysis. It was observed to be homogeneous. The observed N-terminal was only alanine and the molecular weight calculated on the basis of the N-terminal was 35,000. The molecular weight calculated from the sedimentation equiribrium method was 37,000 ± 1,000.

2.) About 320 residues were obtained by using the molecular weight of 37,000, and the amino acid composition was not remarkably changed, when compared it with that of the intact TAA.

## ACKNOWLEDGEMENTS

The author is grateful to Prof. K. Narita and Dr. H. Toda for their kind guidances and valuable discussions through this investigation, to Dr. K. Kakiuchi for his aid to sedimentation measurement, to Mr. Murakami for his helps for amino acid analysis and also to Sankyo Co., Ltd. for their kind supply of " Takadiastase Sankyo ".

## REFERENCES


1.) The first report of this series.

2.) Sanger, F., Biochem. J., 39, 507 ( 1945 )

3.) Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L., Methods of Biochemical analysis, Vol. II, Interscience Publishers, Inc., Nwe York, 1955, p. 359.

4.) Goodwin, T. W., and Morton, R. A., Biochem. J., 40, 628 ( 1946 )

5.) Benesch, R. E., Lardy, H. A., and Benesch, R., J. Biol. Chem., 216, 663 ( 1955 )

6.) Archibald, W. J., J. Physi. Chem., 51, 1204 ( 1947 )

7.) Fuwa, H., J. Biochem., 41, 583 ( 1954 )

8.) Akabori, S., Hagihara, B., and Ikenaka, T., J. Biochem., 41, 577 ( 1954 )

9.) Narita, K., Murakami, H., and Ikenaka, T., will be published.

10.) Lineweaver, H., and Burk, D., L. Am. Chem. Soc., 56, 658 ( 1943 )

11.) Eyring, H., J. Chem. Phys., 3, 107 ( 1953 )

12.) Toda, H., and Akabori, S., J. Biochem., 53, 95 ( 1963 )

13.) Toda, H., J. Biochem., 53, 425 ( 1963 )

14. ) Toda, H., J. Biochem., 54, 1 ( 1963 )

15.) Funatsu, N., and Tokuyasu, K., J. Biochem., 46, 1441 ( ( 1954 )

16.) Okunuki, K., Hagihara, B., Matsubara, H., and Nakayama, T., J. Biochem., 43, 453 ( 1956 )

17.) Graber , P., Advances in Biological and Chemical Physics, 3, pp. 191, Academic Press.

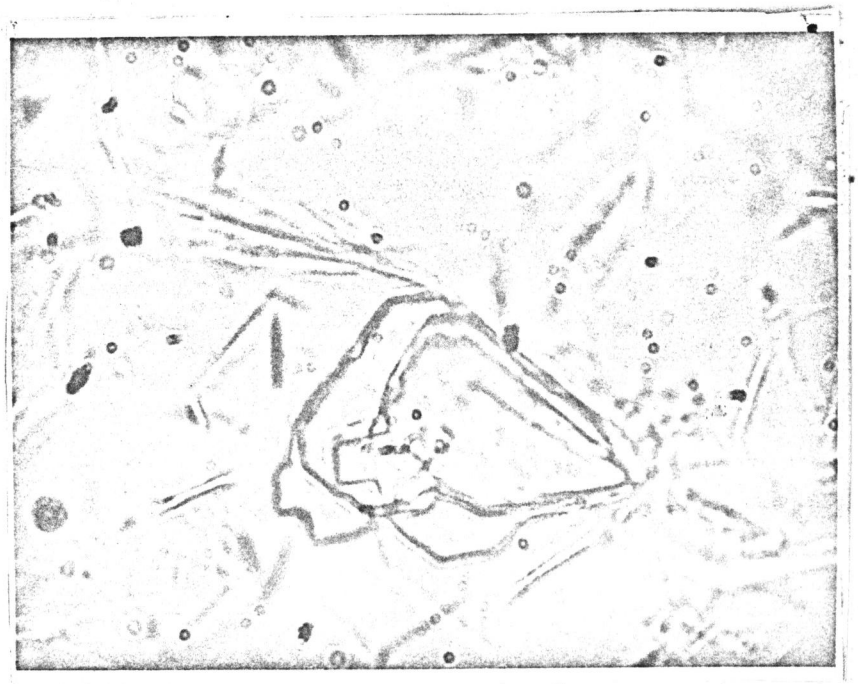


Fig. 1. Photograph of the Crystalline S-TAA-II.

( X 900 )

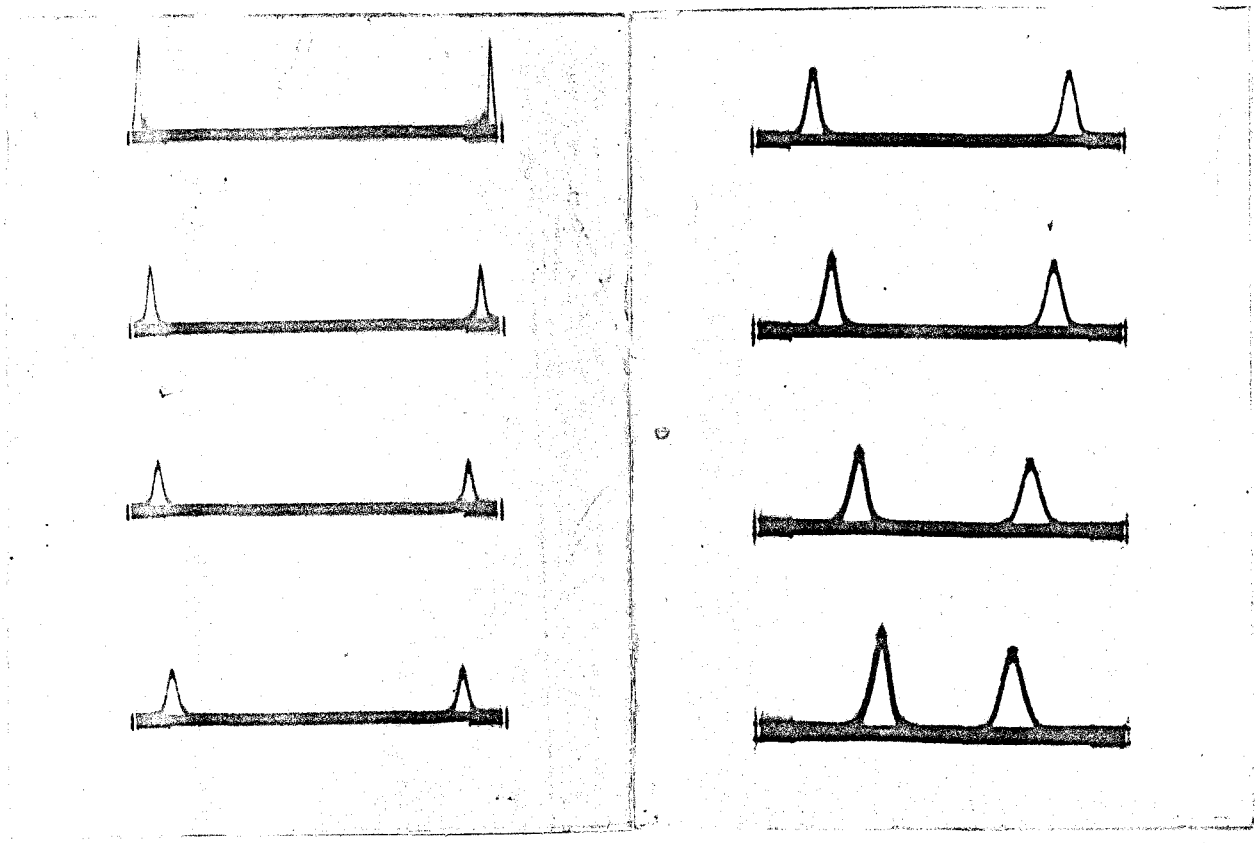


Fig. 2. The Electrophoretic Pattern of the Crystalline S-TAA-II.

pH 5.0, $\mu$ = 0.1 and at 12° C.

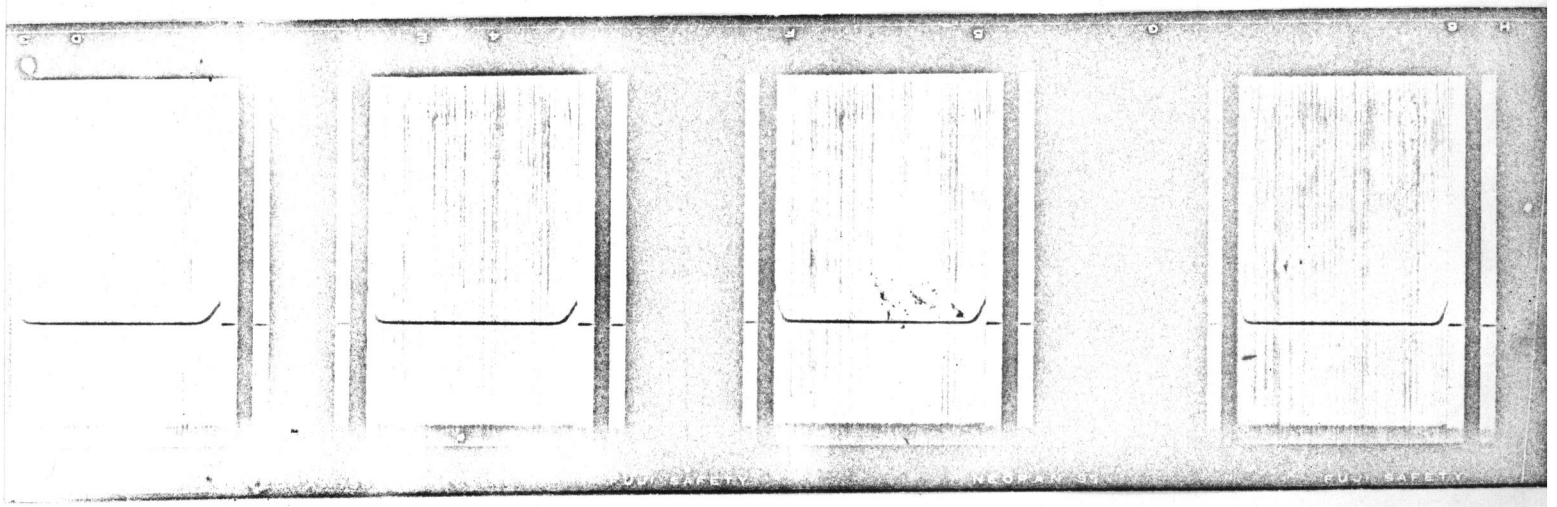


Fig. 3. The Pattern of Archibald Run of the Crystalline S-TAA-II.

1 % aqueous soln. $\mu$ = 0.1 and at 20° C.

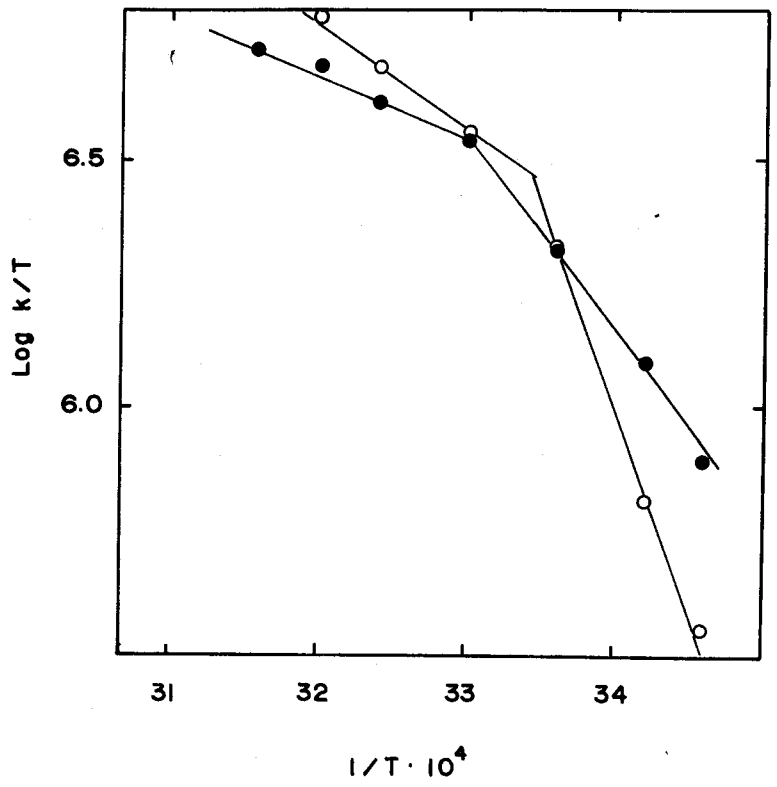



Fig. 4. The Plot of Log K/T versus 1/T of the Crystalline S-TAA-II and the Intact TAA for Amylose.
Assay was performed at pH 5.3, M/5 acetate buffer and 0.3 % amylose.

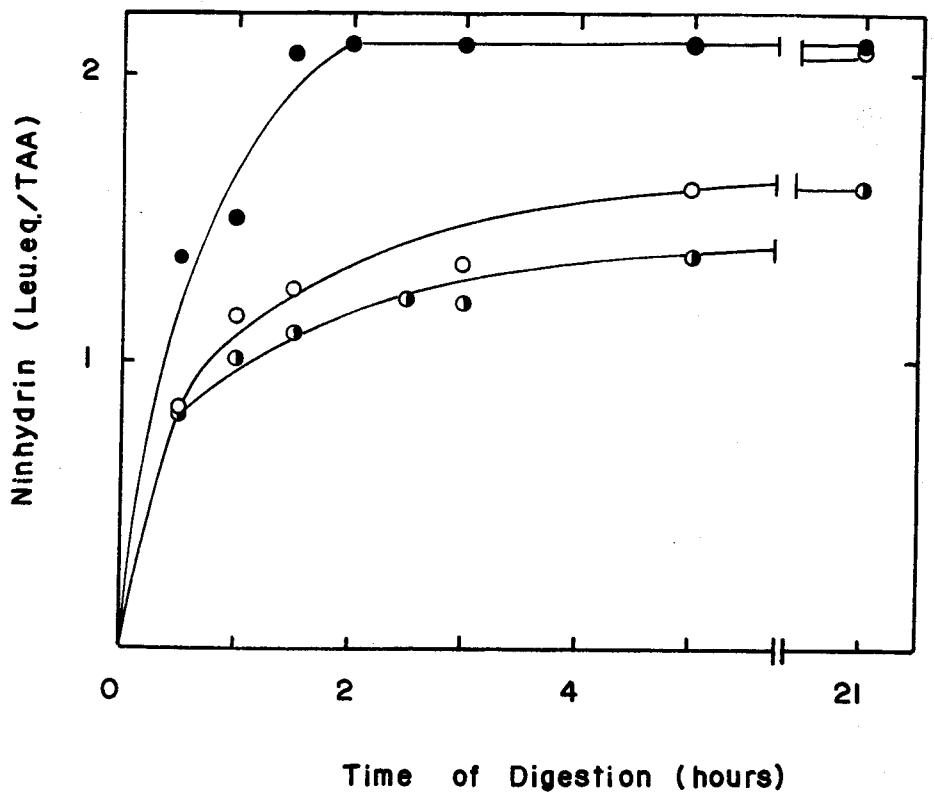



Fig. 5. Time Curves of the Tryptic Digestion of the Crystalline S-TAA-II.

Tryptic digest was performed at pH 8.1, —○— and pH 9.0, —●— and pH 7.4, —◑—, at 20° C.

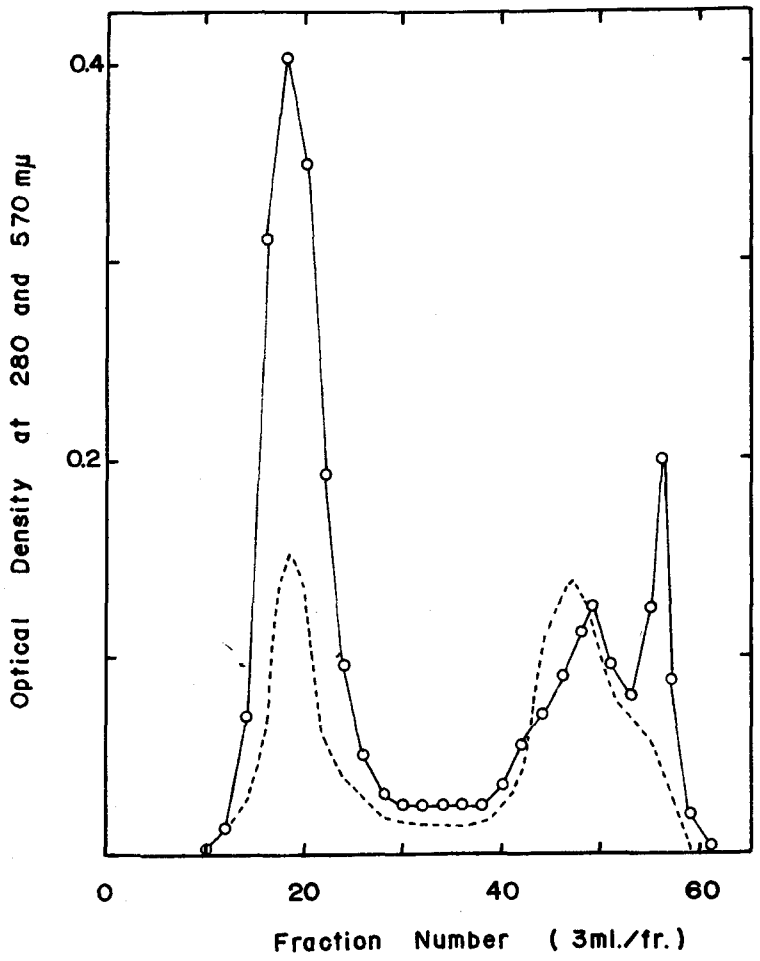



Fig. 6. The Pattern of Gel Filtration of the Tryptically Digested Crystalline S-TAA-II on a Sephadex G 100 Column.

Applied sample was the digest for 3 hours.

—o—, Optical Density at 280 mu and - - -, Optical Density at 570 mu.

Table I.

N-Terminal Group of the Crystalline S-TAA-II.

| Lot Number | Moles of DNP-Alanine per Molee of Crystalline S-TAA-II. |
|---|---|
| 1. | 0.91 |
| 2. | 1.18 |
| 3. | 1.20 |

Above values were obtained assuming its molecular weight as 37,000.

## Table II ( A )

## Amino Acid Composition of the Crystalline S-TAA-II

| Amino Acid | Amino Acid per 100 g. Protein | Amino Acid residue per 100 g. Protein | Minimal Molecular weight |
|---|---|---|---|
| Lysine | 5.50 | 4.83 | 2652 |
| Histidine | 2.19 | 1.94 | 7065 |
| Arginine | 3.21 | 2.88 | 5421 |
| Tryptophane | | | |
| Half-cystine | 1.29 | 1.10 | 9317 |
| Aspartic acid | 17.51 | 15.13 | 760 |
| Methionine | 2.52 | 2.21 | 5934 |
| Threonine | 9.02 | 7.65 | 1321 |
| Serine | 6.98 | 5.78 | 1506 |
| Glutamic acid | 6.74 | 5.91 | 2184 |
| Proline | 4.72 | 3.98 | 2439 |
| Glycine | 6.13 | 4.66 | 1224 |
| Alanine | 6.49 | 5.17 | 1374 |
| Valine | 5.77 | 4.88 | 2030 |
| Isoleucine | 6.12 | 5.28 | 2142 |
| Leucine | 7.97 | 6.87 | 1646 |
| Tyrosine | 9.91 | 8.92 | 1828 |
| Phenylalanine | 4.35 | 3.87 | 3800 |
| Ammonia | 1.47 | 1.38 | |

## Table II ( B )

### Amino Acid Composition of the Crystalline S-TAA-II

| Amino Acid | Calculated No. of residues/molecule, M.W. = 37,000 | No. of residues to nearest integer | Calculated molecular weight |
|---|---|---|---|
| Lysine | 13.95 | 14 | 37.128 |
| Histidine | 5.24 | 5 | 35.325 |
| Arginine | 6.83 | 7 | 37.947 |
| Tryptophane | 7.18 | 7 | |
| Half-cystine | 3.98 | 4 | 37.268 |
| Aspartic acid | 48.68 | 49 | 37.240 |
| Methionine | 6.24 | 6 | 35.604 |
| Threonine | 28.01 | 28 | 36.988 |
| Serine | 24.6 | 25 | 37.650 |
| Glutamic acid | 16.94 | 17 | 37.128 |
| Proline | 15.17 | 15 | 36.585 |
| Glycine | 30.22 | 30 | 36.720 |
| Alanine | 26.92 | 27 | 37.098 |
| Valine | 18.23 | 18 | 36.540 |
| Isoleucine | 17.25 | 17 | 36.414 |
| Leucine | 22.48 | 22 | 36.212 |
| Tyrosine | 20.22 | 20 | 36.560 |
| Phenylalanine | 9.74 | 10 | 38.000 |
| Ammonia | 32.03 | 32 | |
| Total | | | 36.916 ( Average ) |

## Table III ( A )

Comparison of Side Chain Groups in the Crystalline S-TAA II and TAA.

| Type of group, Amino acid | S-TAA-II | TAA |
|---|---|---|
| I. Ionizable side chains | | |
| A. Cationic | | |
| Guanido, arginine | 7 | 9 |
| -Amino, lysine | 14 | 21 |
| Imidazole, histidine | 5 | 7 |
| Total cationic side chains | 26 | 37 |
| B. Anionic | | |
| Carboxyl, aspartic + glutamic acid | 34 | 42 |
| II. Hydrophilic side chanis | | |
| A. Amide, Asparagine + glutamine | 32 | 50 |
| Total carboxyl + amide groups | 66 | 92 |
| B. Hydroxyl | | |
| Aliphatic, serine | 25 | 33 |
| threonine | 28 | 36 |
| Aromatic, tyrosine | 20 | 30 |
| Total hydroxyl groups | 73 | 99 |
| Total nonhydroxyl groups | 165 | 228 |

## Table III ( B )

### Comparison of Side Chain Groups in the Crystalline S-TAA-II and TAA.

| Type of group, Amino acid | | S-TAA-II | TAA |
|---|---|---|---|
| III. | Hydrophobic side chains | | |
| | Hydrogen, glycine | 30 | 38 |
| | Paraffin | | |
| | alanine | 27 | 34 |
| | valine | 18 | 26 |
| | leucine | 22 | 30 |
| | isoleucine | 17 | 24 |
| | Pyrrolidine, proline | 15 | 20 |
| | Aromatic | | |
| | phenylalanine | 10 | 13 |
| | tryptophane | 7 | 10 |
| | Sulfur-containing | | |
| | methoinine | 6 | 8 |
| | half-cystine | 4 | 9 |
| | Total hydrophobic groups | 156 | 212 |
| | Total number of amino acid residues per molecule | 321 | 440 |

Table IV

Kinetic Constants of S-TAA-II and TAA for Amylose

| Kinetic Constants | S-TAA-II | TAA |
|---|---|---|
| $K_m$ | 11.4 mg./ ml. | 32.0 mg. / ml. |
| $V_m$ | 0.8 O.D. / min. | 1.1 O.D. / min. |
| $K_3$ | $1.6 \times 10^6$ O.D./min.M | $4.2 \times 10^6$ O.D./min.M |

## Table V

### Thermodynamic Quantities of the Crystalline S-TAA-II and TAA.

| Thermodynamic quantities | S-TAA-II | TAA |
|---|---|---|
| $\Delta H^{\ddagger}$ ( at low temp. ) | 31.3 K cal. / mole | 17.4 K cal. / mole |
| $\Delta H^{\ddagger}$ ( at high temp. ) | 10.4 | 6.3 |
| $\Delta S^{\ddagger}$ ( at low temp. ) | 79.9 cal./mole deg. | 31.8 cal./mole deg. |
| $\Delta S^{\ddagger}$ ( at high temp. ) | 8.8 | -4.6 |
| $\Delta G^{\ddagger}$ ( at low temp. ) | 7.6 Kcal./mole | 8.4 Kcal./mole |
| $\Delta G^{\ddagger}$ ( at high temp. ) | 6.3 | 7.7 |

These values were obtained from the temperature studies of amylase activity. Crytical temperature for the intact TAA and crystalline S-TAA-II were about 30° C and 25° C, respectively. In this Table, high temp. and low temp. are correspondsng to above and below crytical temperature, respectively.

# Effects of Sonic Oscillation on Taka-Amylase A and Its Derivatives.

## III. Reappearance of Amylase Activity of the Reduced-Carboxymethylated Taka-Amylase A and Its Tryptic Digests by Sonic Oscillation.

By Ikunoshin Kato

# Effects of Sonic Oscillation on Taka-amylase A and Its Derivatives

## III. Reappearance of Amylase Activity of the Reduced-Carboxymethylated Taka-Amylase A and Its Tryptic Digests by Sonic Oscillation.

In the previous papers ( 1 ) and ( 2 ), it was reported that the intact TAA ( Taka-amylase A ) was degraded by sonic oscillation and the crystalline active derivative of molecular weight about 37,000 was isolated as a main product by chromatography on a DEAE-cellulose column and gel filtration on a Sephadex G 75 column. To examine the effects of sonic oscillation on the reduced-carboxymethylated TAA ( R-CM-TAA ), as a control experiment, is the main subject of the present investigation. It was already reported that R-CM-TAA was also degraded in the sonic field and that, the amylase activity, which had once diminished by the reduction of all of the disulfide bridges in the intact TAA and subsequent their carboxymethylation, was revived during sonication ( 1 ). In this paper, the results of further studies on the revivability of amylase activity of R-CM-TAA and of its tryptic digests will be described.

## METHODS AND MATERIALS.

R-CM-TAA. - - - R-CM-TAA was prepared by the method as described in the previous paper ( 1 ).

Trifluoroacetylation of R-CM-TAA.- - - The material was prepared by the method of Anfinsen et al.( 3 ). One per cent of R-CM-TAA and thioethyl tri-fluoroacetate were incubated for 40 min. at pH 9 and room temperature. Resulted trifluoroacetylated derivative was precipitated by cold methanol, of which final concentration was 60 %. The precipitate was collected by centrifuge and dissolved in small volume of deionized water for 2 - 3 days in a cold room and the dialysate was lyophilized ( R-CM-TF-TAA )

Trypsin.- - - Three times recrystallized trypsin was kindly supplied by Dr. Vanecek. Chymotrypsin contaminating in this trypsin was less than 0.2 %.

Tryptic Digestion of R-CM- and R-CM-TF-TAA.

Tryptic digestions of the both proteins were carried out at pH 8.0 and room temperature following the reaction with NaOH by using a pH stat, Radiometer, for 8 - 10 hours. Two per cent ( W/W ) of trypsin for protein was used.

Sonic Oscillation.- - - Sonic oscillation was performed by sonic sonic oscillator, Kubota type KMS 100 of 10 KC., and 100 W. The applied volume was kept as 15 ml. and the protein solution was always kept at 0 - 3° C by circulating ice water.

Amylase Activity.- - - The activity was measured by sacharifing power for amylose ( Nagase ), according to the Fuwa's method ( 4 ).

Amino Acid Analysis.- - - The analysis was carried out by using an automatic amino acid analyzer, Beckman Spinco. The estimation of S-carboxymethyl-cysteine was performed according to the Moore's method ( 5 ).

## EXPERIMENTS AND RESULTS

### Time curves of Sonic Oscillation on R-CM-TAA.

As described in the previous paper ( 1 ), R-CM-TAA was degraded by sonic Oscillation and the number of peptide bond cleaved was about 7 per mole of R-CM-TAA. The reduced viscosity decreased by sonication. However, the once diminished amylase activity was revived. The results are presented in Fig. 1.

- - - - - - - - - - - - - - - - -

Fig. 1 . Time Curves of Sonic Oscillation on R-CM-TAA.

- - - - - - - - - - - - - - - - -

To discard enzymatically inactive products, sonicated product of R-CM-TAA was subjected to gel filtration on a column of Sephadex G 100, as shown in Fig. 2.

The first enzymatically active peak in the chromatogram was eluted out at the position of the oribinal R-CM-TAA

and this was collected to use for the sedimentation analysis. As shown in Fig. 3, two main peaks were observed in the sedimentation pattern.

- - - - - - - - - - - - - - - - - -

Fig. 2. Elution Curves of the Sonicated R-CM-TAA for 180 min. from a column of Sephadex G 100.

- - - - - - - - - - - - - - - - - -

- - - - - - - - - - - - - - - - - -

Fig. 3. Sedimentation Pattern of the Sonicated enzymatically active derivative.

- - - - - - - - - - - - - - - - - -

Thus the observed revivability of amylase activity leads to the following two posibilityes ; ( 1 ) The formation of the disulfide bridges in R-CM-TAA by the sonication occurred, or ( 2 ) some other comformations were formed. Therfore, the former possibility, reformation of the disulfide bonds, was examined. The above active material obtained was subjected to an automatic amino acid analyzer to estimate the amount of S-CM-cysteine residues. The results were summarized in Table I.

---

Table I. The Analysis of S-CM-cysteine in the Enzymatically Active Derivative of Sonicated R-CM-TAA.

---

The results showsd that this active material contained all of the S-CM-cysteine and no disulfide bridges were formed by sonic oscillation.

The enzymatic activities for amylose of the sonicated R-CM-TAA and of its purified fraction were compared with that of the intact TAA, as listed in Table II. The values of before and after purification were aproximately 2 and 15 % of the intact TAA, respectively.

---

Table II. Comparison of Amylase Activity of the Sonicated R-CM-TAA with that of the Intact TAA.

---

Requirements for the Revival of Amylase Activity of R-CM-TAA.

It is well established that $Ca^{++}$ is necessary for the amylase activity ( 6 ), but, it is not known yet whether $Ca^{++}$ ion is required or not for the revival of amylase activity from R-CM-TAA. Then, the effect of concentration of $Ca^{++}$ ion was examined by using calcium acetate and the results are shown in Fig.4.

- - - - - - - - - - - - - - - - - - -

Fig. 4. Effect of Calcium Ion on the Revivability of the Amylase Activity.

- - - - - - - - - - - - - - - - - - -

The optimal concentration of $Ca^{++}$ ion for this reaction seems to be about $10^{-3}$ M.

At the second, the pH dependence was examined with the system of $10^{-3}$ M $Ca^{++}$ and 0.2 % R-CM-TAA. The pH of the solution was adjusted at the desired pH with 0.1 N HCl and NaOH and the results are presented in Fig. 5.

- - - - - - - - - - - - - - - - - - -

Fig.5. The pH Dependence of the Revivability of Amylase Activity.

- - - - - - - - - - - - - - - - - - -

The results indicate that the optimum pH for this reaction was about 7.

As a third factor, the effect of concentration of R-CM-TAA was examined at pH 7.0 and in the presence of $10^{-3}$ M $Ca^{++}$ ion. The results showed that dilute solution of 0.1 - 0.2 % were the most suitable for the revivality of the enzyme activity as presented in Fig. 6.

- - - - - - - - - - - - - - - -

Fig. 6. The Effect of the Concentration of R-CM-TAA for the revivability of the Activity.

- - - - - - - - - - - - - - - -

Finally, the effect of ionic strength was examined by using potassium chloride. A remarkable effect was observed at the region of high ionic strength, $\mu = 1.0$, and was shown in Fig. 7.

- - - - - - - - - - - - - - - - - - -

Fig. 7. The Effect of Ionic Strength.

- - - - - - - - - - - - - - - - - -

The Revivability of Amylase Activity of the Tryptic Digests of R-CM-TAA. and R-CM-TF-TAA.

As previuosly described, R-CM-TAA became to have amylase activity by sonic oscillation under the conditions used, namely at pH 7.0, in the presence of $10^{-3}$ M $Ca^{++}$ ion and the use of 0.1 - 0.2 % of R-CM-TAA. It was also shown that the revived amylase activity was not due to the regeneration of some disulfide bridges.

Therfore, similar results were expected even in the case of the tryptic digests of R-CM-TAA which have no disulfide bridges and which lost the original primary structure in considerable extent. Then, the revivability of the activity of the two tryptic digests of R-CM-TAA and R-CM-TF-TAA were examined under the optimum conditions for R-CM-TAA

respectively. The results for the both digests were met for the expectation, but the extent of the revived activity was different from that of the sonicated R-CM-TAA. The former was aproximately one tenth of the sonicated R-CM-TAA and the latter was close to the same. These results were presented in Fig.8.

- - - - - - - - - - - - - - - - - -

Fig. 8. The Revived Activities of R-CM- and R-CM-TF-TAA and their tryptic digests by Sonic Vibration as a Function of Time.

- - - - - - - - - - - - - - - - -

These two kinds of tryptic digests, designated as R-CM-TD and R-TF-TD, were examined on the chromatographic behaviors in gel filtration on a Sephadex G 100 column, before and after sonication, respectively, and the chromatograms are shown in Figs. 9 and 10.

- - - - - - - - - - - - - - - - - - -

Fig. 9. Chromatogram of R-CM-TD before and after Sonication on a column of Sephadex G 100.

- - - - - - - - - - - - - - - - - - -

- - - - - - - - - - - - - - - - - - -

Fig.10. Chromatogram of R-TF-TD before and after Sonication on a Column of Sephdex G 100.

- - - - - - - - - - - - - - - - - - -

In the case of R-TF-TD, the main peak from a Sephadex column of the sonicated sample eluted remarkably fast comparing the position of the peak of the sample before sonication. The breakthrough peak have amylase activity

but the slow moving peaks have not. The position of first active peak nearly corresponds to that of the original r-TF+TAA.

On the other hands, one third of the main peak of R-CM-TD migrated to forward position at elution volume by sonication. The activity was observed in the forward parts of elution diagram.

Group Separation of Two Tryptic Digests, R-CM-TD and R-TF TD, and Thier Reconstitution by Sonic Oscillation.

It is not known from the above experiments mentioned whether the revivability of amylase action of the two tryptic digests are due to the structural reorganization of one peptide or a associate of the several peptides. Therfore, at first the group separations of two kinds of tryptic digests were performed by gel filtration on columns of Sephadex G25 and 50.

- - - - - - - - - - - - - - - - -

Fig. 11 ( a ). The Group Separation of R-CM-TD on a Sephadex G 25 Column.

- - - - - - - - - - - - - - - - - -

R-CM-TD was placed on the top of a Sephadex G 25 column and was separate into two groups as shown in Fig. 11 ( a ).

The first peak was further fractionated into three peaks by gel filtration on a column of Sephadex G 50 as represented in Fig. 11 ( b ). In order of the elution of the four peaks, they were designated as P 1, P 2, P 3, and P 4, respectively.

- - - - - - - - - - - - - - - - - -

Fig. 11 ( b ). Further Fractionation of the First Peak Separated by a Sephadex G 25 Column ( Fig. 11 ( a ) ) onaa Sephdex G 50 Column.

- - - - - - - - - - - - - - - - - -

Similarly, R-TF-TD was also subjected to gel filtration on a Sephadex G 25 column but only one peak was obtained as shown in Fig. 12 ( a ). Therfore, it was agin fractionated on a Sephadex G 50 column and four peaks were obtained as

represented in Fig. 12 ( b ). These four peaks were also designated as P 1, P 2, P 3 and P 4, respectively.

- - - - - - - - - - - - - - - - - - -

Fig. 12 ( a ). The Pattern of Gel Filtration of R-TF-TD on a Sephadex G 25 Column.

- - - - - - - - - - - - - - - - - - -

- - - - - - - - - - - - - - - - - - -

Fig. 12 ( b ). The Pattern of Gel Filtration of R-TF-TD on a Sephadex G 50 Column.

- - - - - - - - - - - - - - - - - - -

As the second, to determine the essential peptide group

for the revival of amylase activity, possible combinations of three groups were sonicated for the two kind of tryptic digests, respectively. The possible all three group combinations are as follows ; 1-2-3, 2-3-4, 3-4-1 and 4-1-2. Deionized water was substituted for the defect fraction in each combination and three groups were mixed as become of 0.2 % system of full TD solution. The sonic oscillation was performed under the previously determined optimal conditions. The results of R-CM-TD combination are shown in Fig. 13.

- - - - - - - - - - - - - - - - - -

Fig. 13. The Activity Time Curves of four Combinations of Peptide Groups of R-CM-TD.

- - - - - - - - - - - - - - - - - -

The positive combinations to produce amylase activity were the combinations of 1-2-3 and 2-3-4 except complete system. Therfore the combination of the peptide fractions

of 2 and 3 appears to be essential for revival of the amylase activity by sonic oscillation.

In turn, all combinations of three groups out of the four peptide fractions were negative in the case of R-TF-TD except the complete system as represented in Fig. 14. Therefore it is evident in this case that the four peptide fractions are required for the revival of amylase activity.

- - - - - - - - - - - - - - - - - -

Fig. 14. The Activity Time Curves of Combinations of Peptide Groups of R-TF-TD.

- - - - - - - - - - - - - - - - - -

## DISCUSSION.

In 1963. Asakura et al. ( 7 ) reported that sonic wave has some organizing action on actin molecule and they indicated that the effects of sonic oscillation are the induction of intermolecular interaction among actin molecules.

As described in proceeding paper ( 1, 2 ), we also applied the energy of sonic oscillation to the degradation of the intact TAA molecule. As the second step, R-CM-TAA, which does not posess any high organized conformation, was also subjected to sonic oscillation as a control experiment and some unexpected results could be obtained.

R-CM-TAA was degraded as was expected on the basis of the proceeding studies on the intact TAA ( 1 ), but astonishingly once diminished amylase activity has appeared by sonic vibration as represented in Fig. 1. As shown in Figs. 1 and 2, the results of purification of the sonicated R-CM-TAA by gel filtration suggest that the active product seems to have nearly the same molecular size as the original R-CM-TAA. This result was also supported by the sedimentation analysis as shown in Fig. 3. Assuming the active product has about the same molecular weight, the amino acid analysis indicate that it contained approximately entire S-CM-cysteine

reisdues of the original R-CM-TAA. This fact ( Table I ) ruled out the possibility that the revived amylase activity are due to the reformation of the disulfide linkages of the intact TAA as studied by Isemura et al. ( 8 ).

Although the estimation of $Ca^{++}$ ion content was not carried out , it seems that starting material of R-CM-TAA does not contain any $Ca^{++}$ ion considering the preparation procedures of R-CM-TAA. Therefore it is expected that the revaivability of the enzymatic activity depends on $Ca^{++}$ ion content as studied by Oikawa et al. ( 5 ) with the intact TAA. The above considerations were proved to be probable as shown in Fig. 4.

Thus we propose the following mechanism for the phenomenon of the revival of the enzymatic activity from the denatured enzyme, R-CM-TAA. $Ca^{++}$ will play the central role to attract the essential parts inthe polypeptide chain to form an active site and sonic vibration will provide energy to produce secondary linkages between amino acid residues in the peptide leading to stabilization of the active site. Thus it will be supposed that the pH dependency of the revival reaction will be remarkable since the pH will affect both the state of calcium ion and of the

functional dissociable groups which will act as conbinders with other parts in the peptide chain.

The effect of concentration suggests that the revivability are due to the intra peptide reaction and threfore it was supposed that undesired reaction, intermolecular interaction, will be occurred in high concentration.

Judging from the above experiments and from the probable mechanism for the revivability of the amylase activity of R-CM-TAA, it is considered that if the residues constituting catalytic site of active center remains unchanged even after sonic oscillation, similar results can be expected by using the tryptic digests of R-CM-TAA and R-CM-TF-TAA under the same concentration those for sonication of the R-CM-TAA. As might have been expected, the revival of amylase activity of the two kinds of tryptic digests were observed as represented in Fig. 8.

Comparing with the control pattern of gel filtration of R-CM-TD and R-TF-TD, the patterns of their sonicated products of 30 min.' sonication show that the association among several peptides have occurred by the sonication and the resulted associates have amylase activity. However, the extent of revivability for two kinds of tryptic digests, R-CM-TD and

R-TF-TD, was different from each other.

Theoretically, R-TF-TAA, which have nine arginine residues, are fragmentated into ten peptides, and R-CM-TAA, which have susceptible nine arginine + twenty one lysine residues, are degraded into thirty one peptides by the complete tryptic digestion.

Then, it may be considered that the encountering probability of the peptides, which construct the active center, are tightly related to the numbers of fragmentated peptides and to the extent of revived amylase activity.

However, it is almost unkown whether the revivability of the tryptic peptides of the both modified amylase will depends on the association of several peptides or will depend on the reconstruction of the active comformation of only one peptide.

The results ( Fig. 13 and 14 ) indicated that the revivability of amylase activity was attributed to at least two peptides, 2 and 3 groups in R-CM-TD, and four peptides in R-TF-TD, respectively.

As a conclusion, by sonic oscillation, the peptides which constitute an active center of the intact TAA, reconstituted an active fragment possessing some comformations.

However, whether the conformation of the reconstituted active site is similar to that of the intact TAA or not and what kinds of linkages have formed without the disulfide bridges are beyond our speculation at this state of the studies. These will be discussed in the subsequent paper.

Finally, from the above experiments, a probable scheme for the action of sonic vibration on the modified enzymes and their tryptic digests are shown in Scheme I.

- - - - - - - - - - - - - - - -

Scheme I. A Probable Scheme for the Mechanism of the Revivability of Amylase Activity by Sonic Oscillation.

- - - - - - - - - - - - - - - - - -

## SUMMARY.

1.) R-CM-TAA which possesses no disulfide bridges revived

its amylase activity by the sonic oscillation under the conditions of 10 KC., $0^{\circ}$ C, pH 7.0 , $10^{-3}$ M $Ca^{++}$ ion and 0.2 % of R-CM-TAA. The revived specific amylase activity was about 15 % of that of the intact TAA.

2.) The two kinds of tryptic digests, R-CM-TD and R-TF-TD, were also shown to revive their amylase activities under the same conditions as for R-CM-TAA.

3.) It was indicated that the revived amylase activities of the two tryptic digests are due to the association among several peptides fractionated by gel filtration ona Sephadex G 100 column.


## ACKNOWLEDGEMENTS

Author wish to express his thanks to Prof. K. Narita and to Dr. H. Toda for their gudances and discussions through this investigation and also to Sankyo Co., Ltd. for their kind supply of " Takadiastase Sankyo ".


## REFERENCES.


1.) The first report of this series.

2.) The second report of this series.

3.) Goldberger,R.F., and Anfinsen,C.B.,Biochem.,1, 401 ( 1962 )

4.) Fuwa, H., J. Biochem., 41, 583 ( 1954 )

## Table I

Amino Acid Composition of the Active Fraction of S-CM-TAA for 180 min.' Sonication.

| Amino Acid | Amino acid residues per 100 g. protein | minimal molecular weight | Calculated No. of residues/molecule. M.W. = 51,000 |
|---|---|---|---|
| Lysine | 4.54 | 2,821 | 18.3 |
| Histidine | 1.89 | 7,252 | 6.9 |
| Arginine | 3.10 | 5,036 | 10.1 |
| S-carboxymethyl-cysteine | 2.20 | 7318 | 7.00 |
| Aspartic acid | 15.76 | 730 | 69.9 |
| Threonine | 8.27 | 1,222 | 42.1 |
| Serine | 4.83 | 1,802 | 28.3 |
| Glutamic acid | 8.31 | 1,553 | 32.8 |
| plroline | 3.93 | 2,470 | 20.6 |
| Glycine | 5.08 | 1,123 | 45.3 |
| Alanine | 5.53 | 1285 | 39.7 |
| Half-cystine | — | — | — |
| Valine | 5.06 | 1,958 | 26.1 |
| Methionine | 1.95 | 6724 | 7.5 |
| Isoleucine | 5.62 | 2,012 | 25.3 |
| Leucine | 6.39 | 1,770 | 28.7 |
| Tyrosine | 8.64 | 1,887 | 27.0 |

Table II

Comparison of Amylase Activity of the sonicated R-CM-TAA with that of the Intact TAA.

| Protein | Time of Sonicn. ( min. ) | Specific Activity ( liter/min. ) | Relativity ( % ) |
|---|---|---|---|
| TAA | 0 | $74.0 \times 10^{-1}$ | 100 |
| Crude Sonicated R-CM-TAA | 60 | $1.6 \times 10^{-1}$ | 2.2 |
| Purified S-R-CM-TAA | 180 | $11.2 \times 10^{-1}$ | 15.1 |

Sonifation was performed at the conditions of the presence of $10^{-3}$ M $Ca^{++}$, at neutral and 0.2 % of R-CM-TAA.

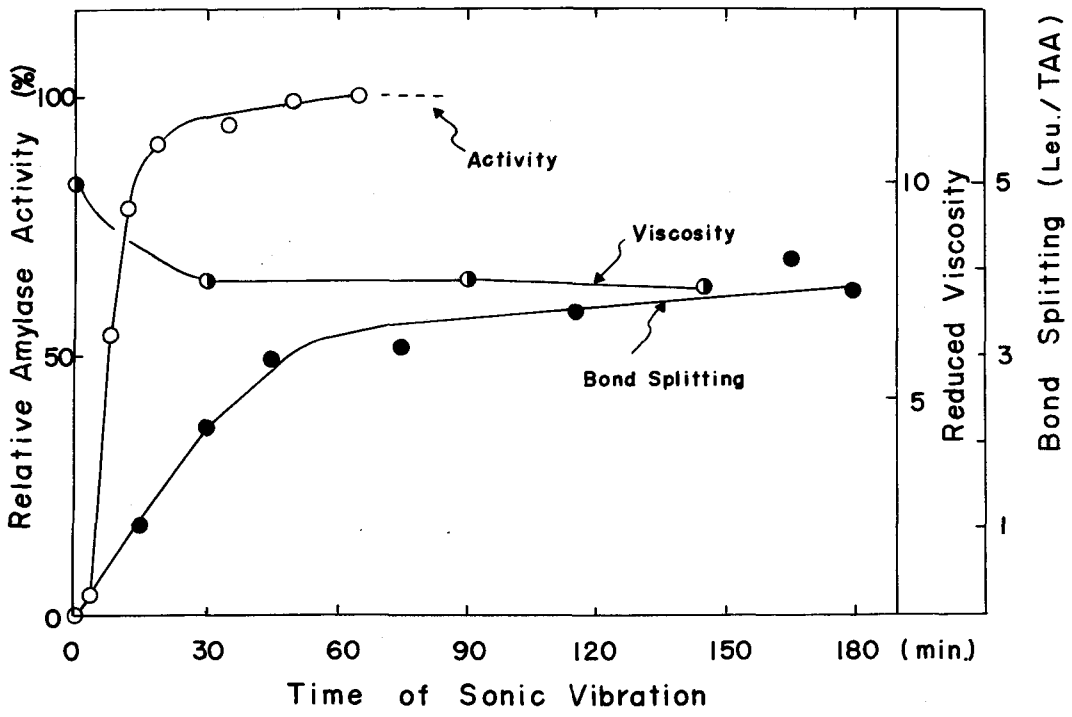



Fig. 1, Time Curves of Sonic Oscillation of R-CM-TAA.
The conditions of sonication ; 15 ml. of 0.56 % aqueous solution of R-CM-TAA containning $10^{-3}$M $Ca^{++}$.

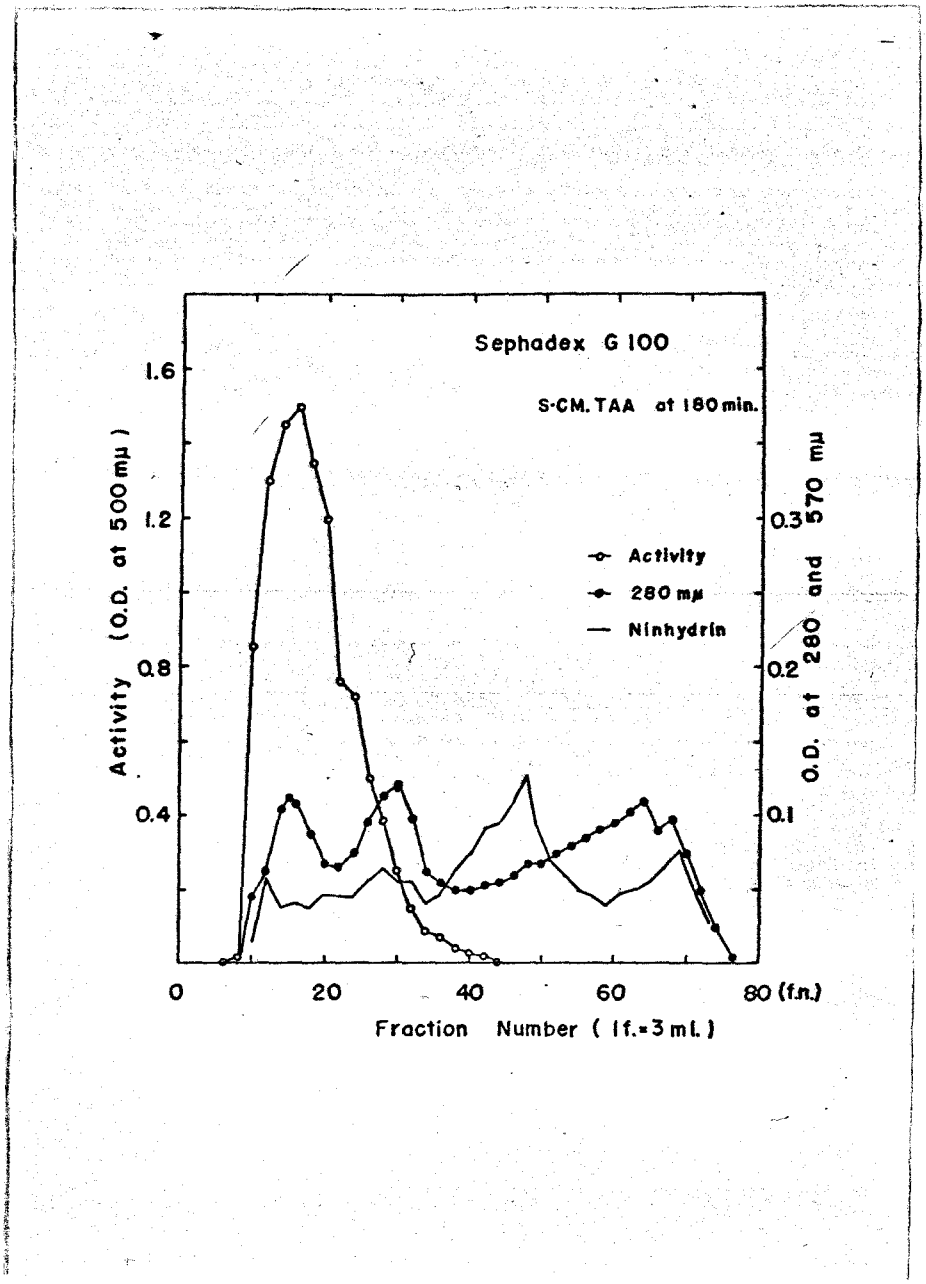



Fig. 2, The Pattern of Gel Filtration of 180 min. sonicated R-CM-TAA on a Sephadex G 100 Column ( 3 × 17 cm. ). —o—, amylase activity expressed as optical density at 500 mu ; —●—, optical density at 280 mu ; ——, optical density at 570 mu.

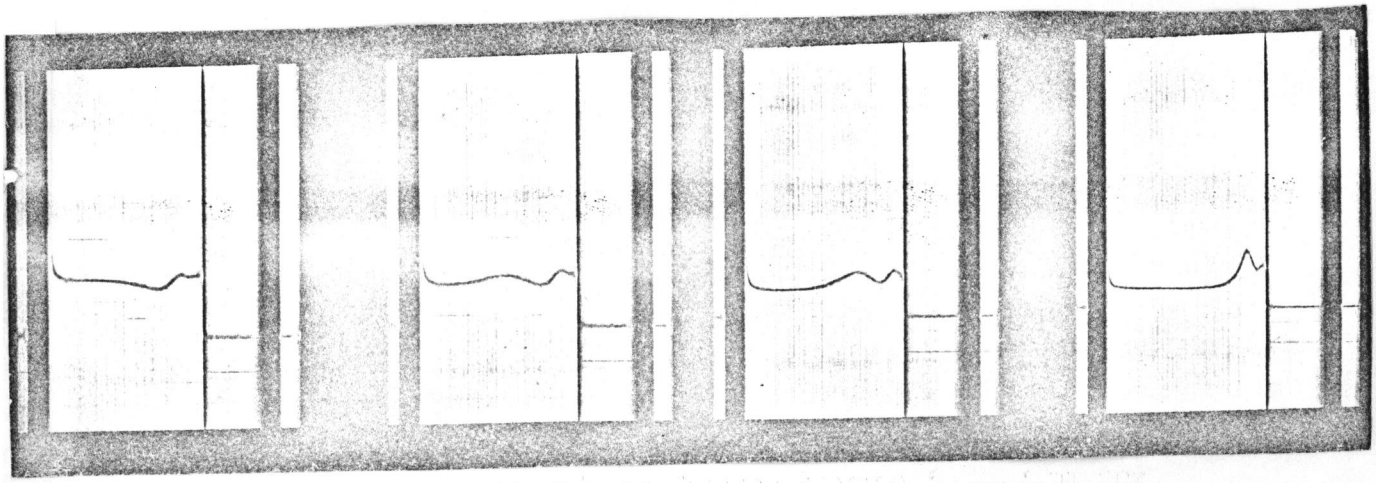


Fig. 3. Sedimentation Pattern of the Sonicated Derivative of R-CM-TAA.

1 % and $\mu = 0.1$.

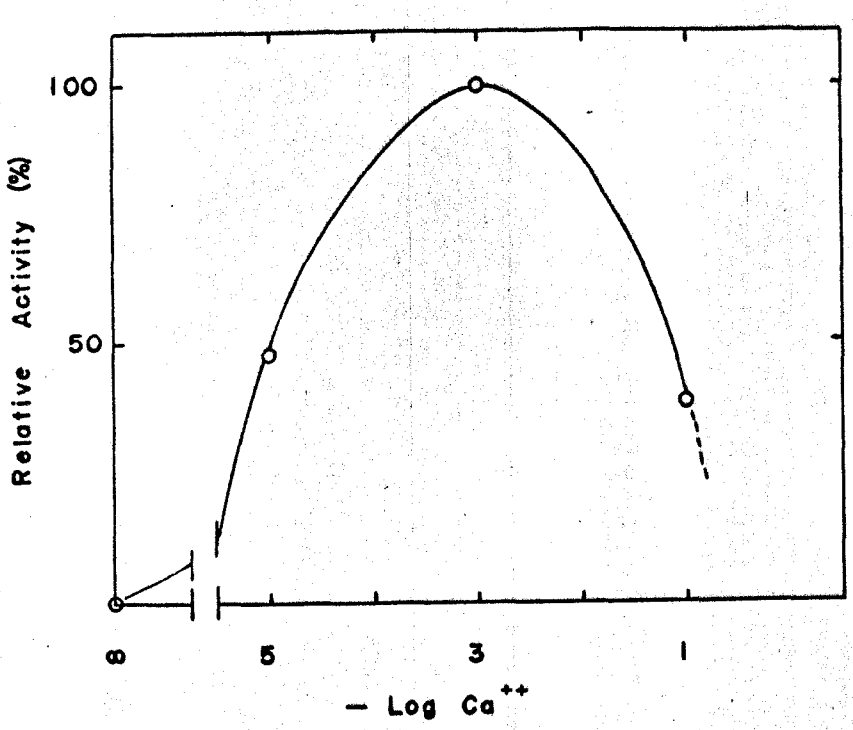



Fig. 4, Effect of Calcium Ion on the Revivalility of Amylase Activity.

Sonication conditions : 0.2 % of R-CM-TAA, pH 7, and 30 min. sonication.

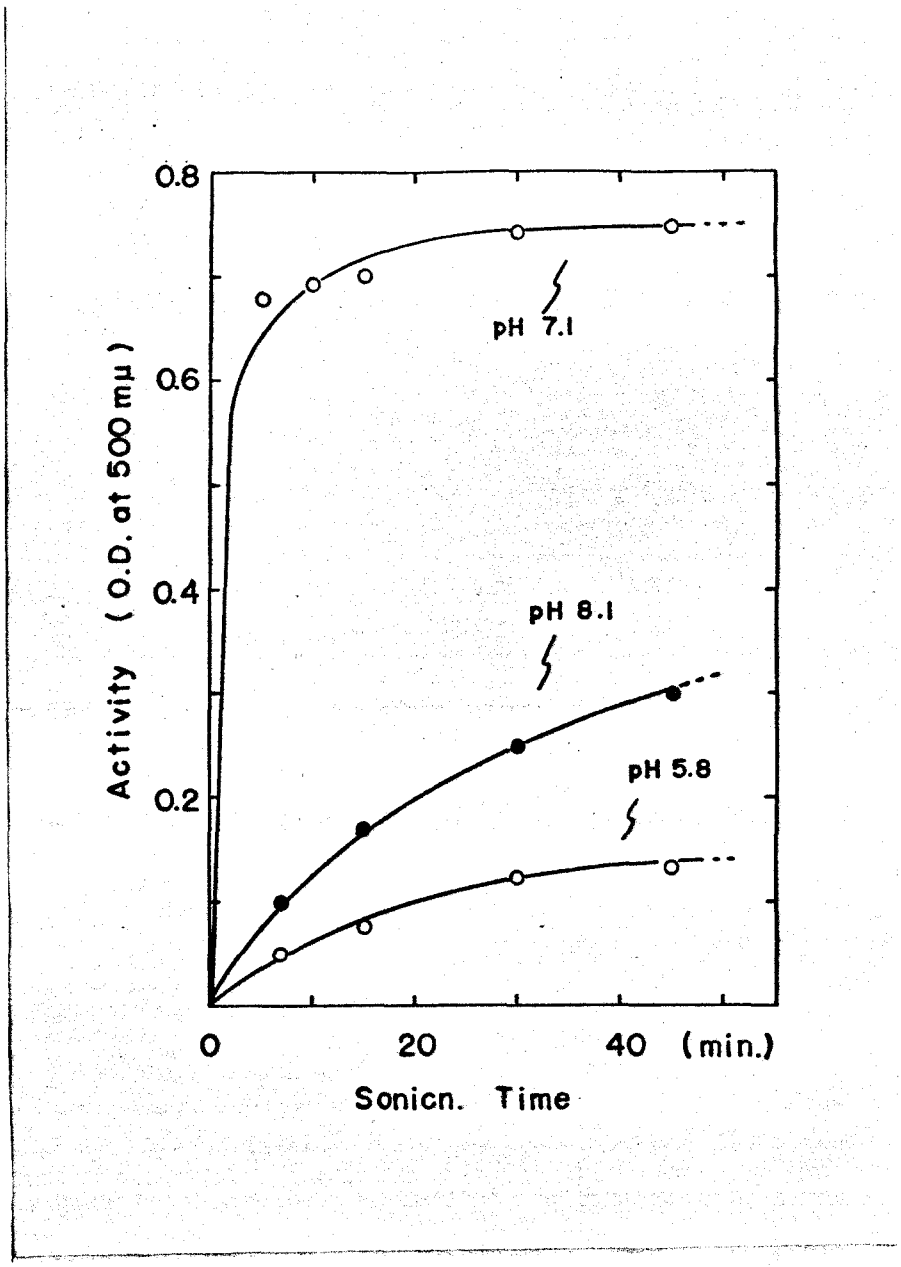



Fig. 5. PH Dependence of the Revivability of Amylase Activity.
Sonication Conditions : 0.2 % R-CM-TAA and $10^{-3}$ M $Ca^{++}$.
PH was adjusted with HCl and NaOH.

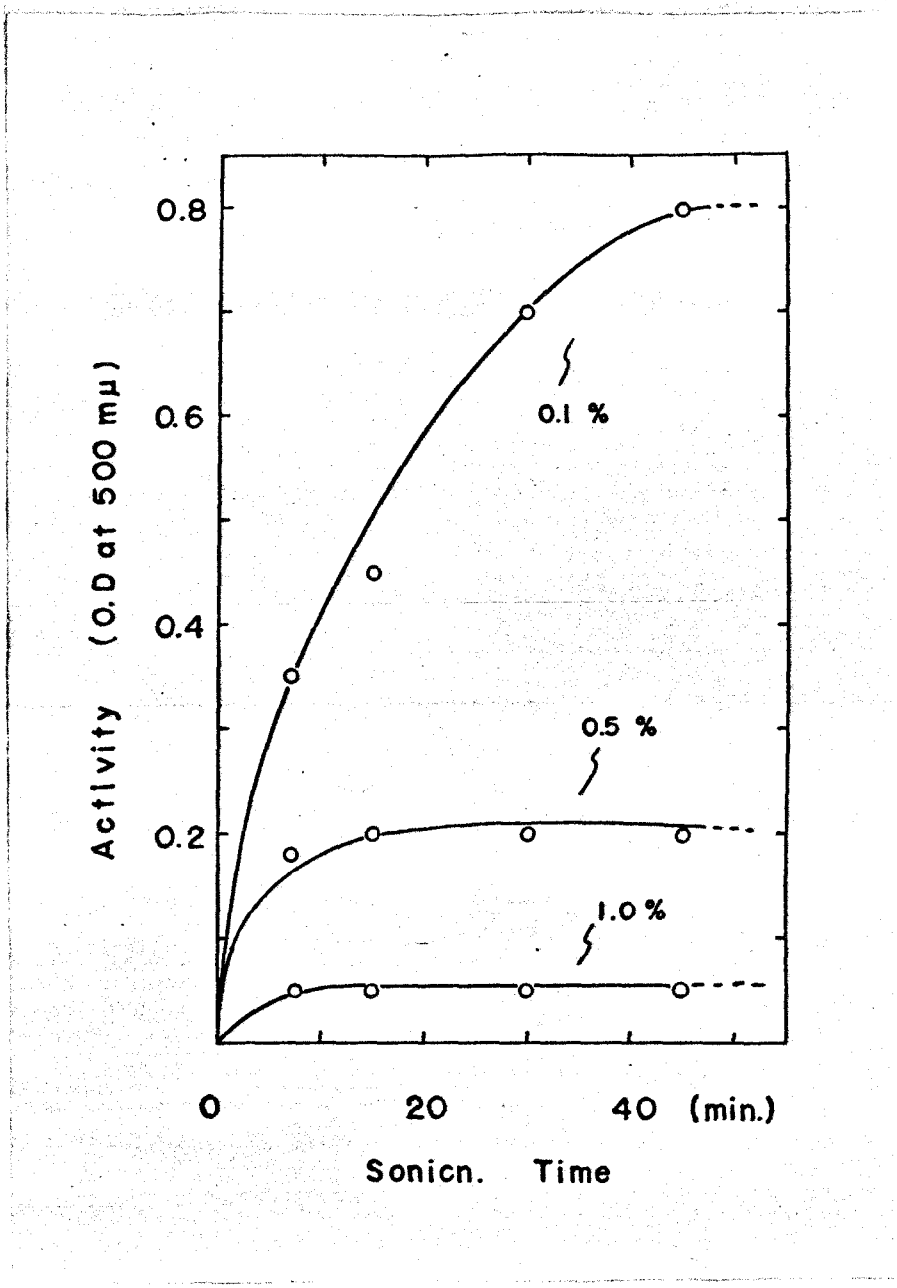



Fig. 6, The Effect of R-CM-TAA Concentration for the Revivability of Amylase Activity.

Sonication conditions : PH 7, $10^{-3}$ M $Ca^{++}$.

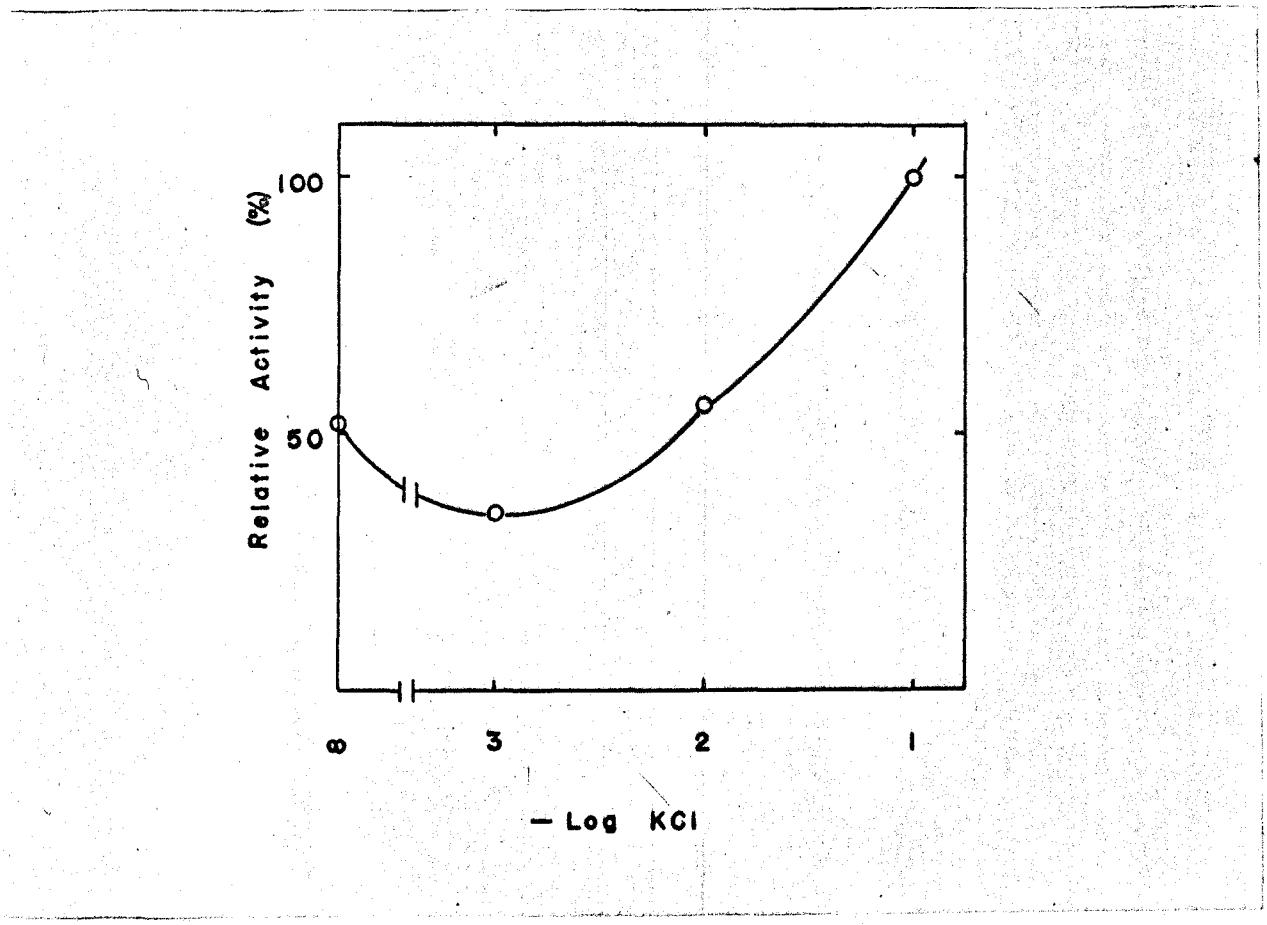



Fig. 7. The Effect of Ionic Strength on the Revivability of Amylase Activity.

Sonication Conditions : pH 7, $10^{-3}$ M $Ca^{++}$ , 0.2 % of R-CM-TAA.

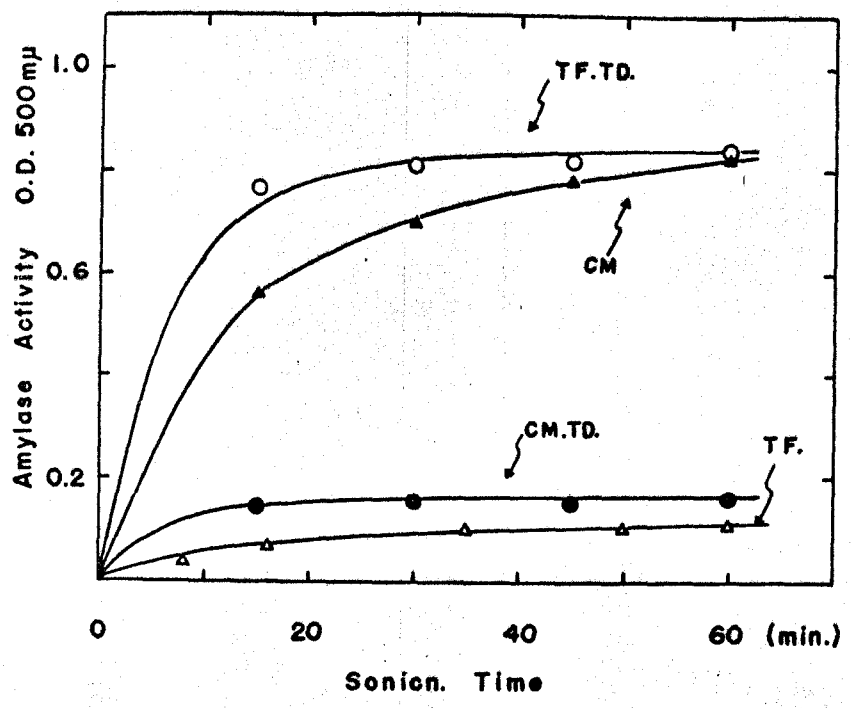



Fig. 8. The Time Curves of the Revivability of Amylase Activity of R-CM-TAA and Its Derivatives.

Sonication conditions : 0.2 % of R-CM-TAA, $10^{-3}$ M M $Ca^{++}$ and pH 7.

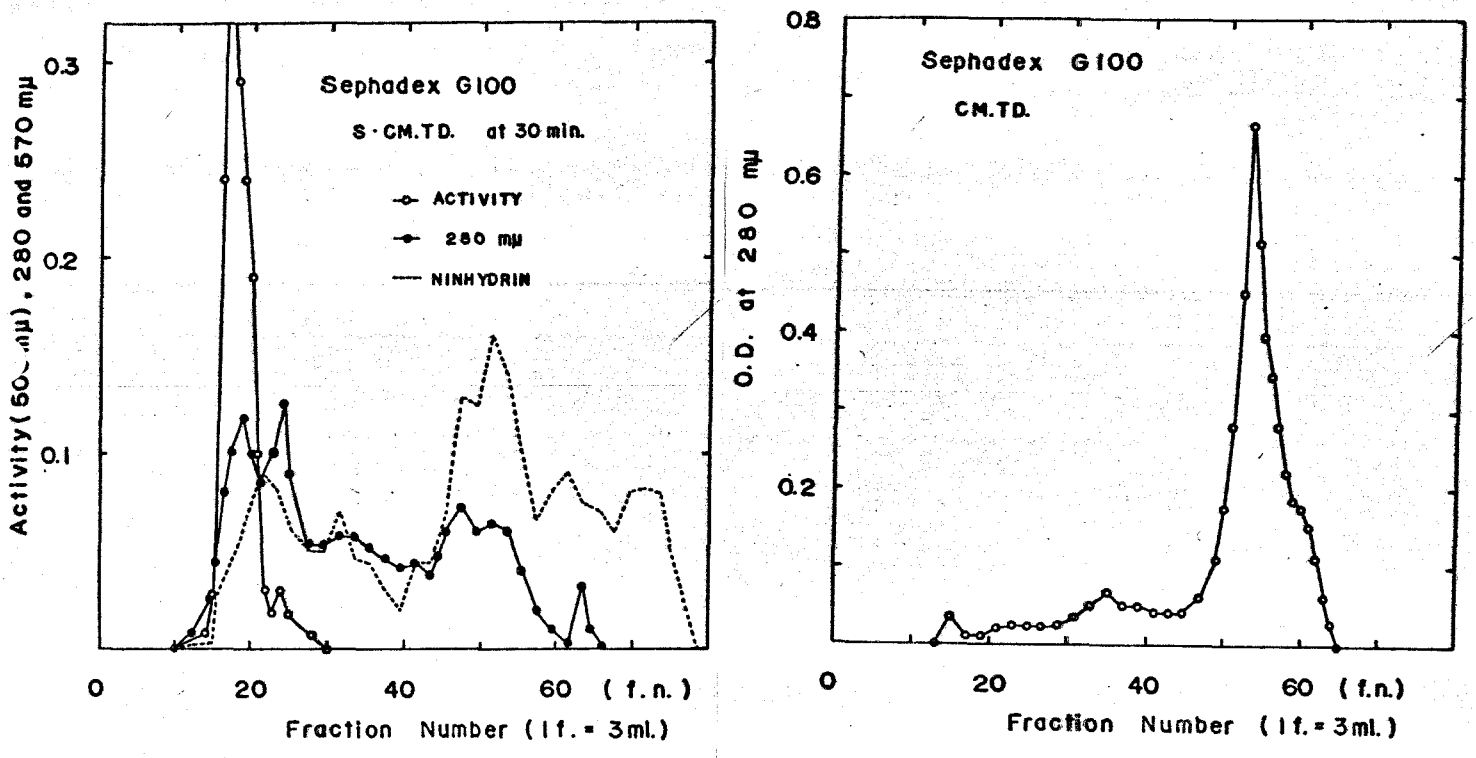



Fig. 9. The Pattern of Gel Filtration of R-CM-TD before and after Sonication on a Sephadex G 100 Column ( 3 X 50 cm. ).

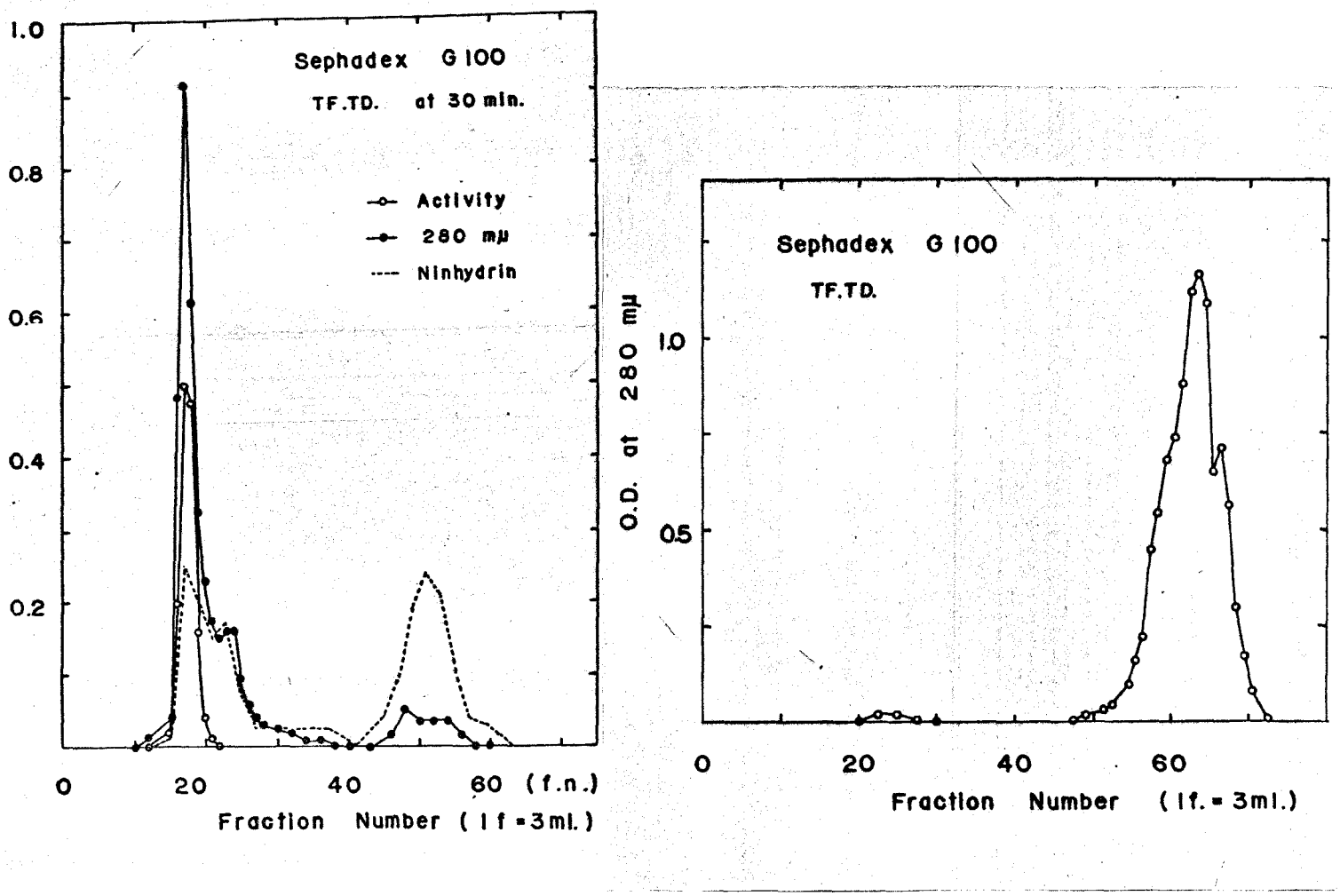



Fig. 10. The Pattern of Gel Filtration of R-TF-TD before and after Sonication on a Sephadex G 100 Column ( 3 X 50 cm. ).

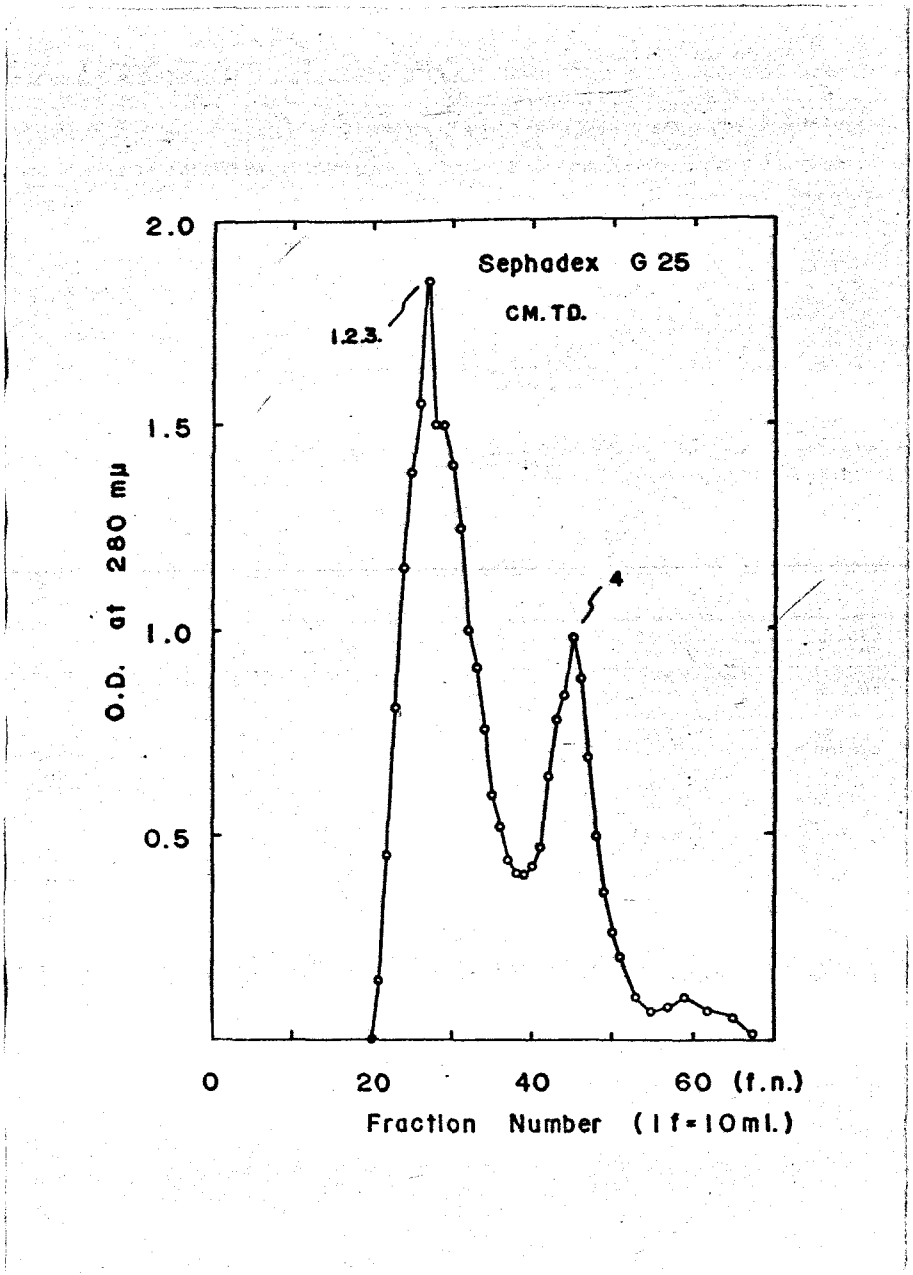



Fig. 11. ( a ). Group Separation of R-CM-TD by Gel Filtration on a Sephadex G 25 Column ( 5 X 30 cm. ).
One per cent solution of the tryptic digest for 10 hours at room temperature was applied.

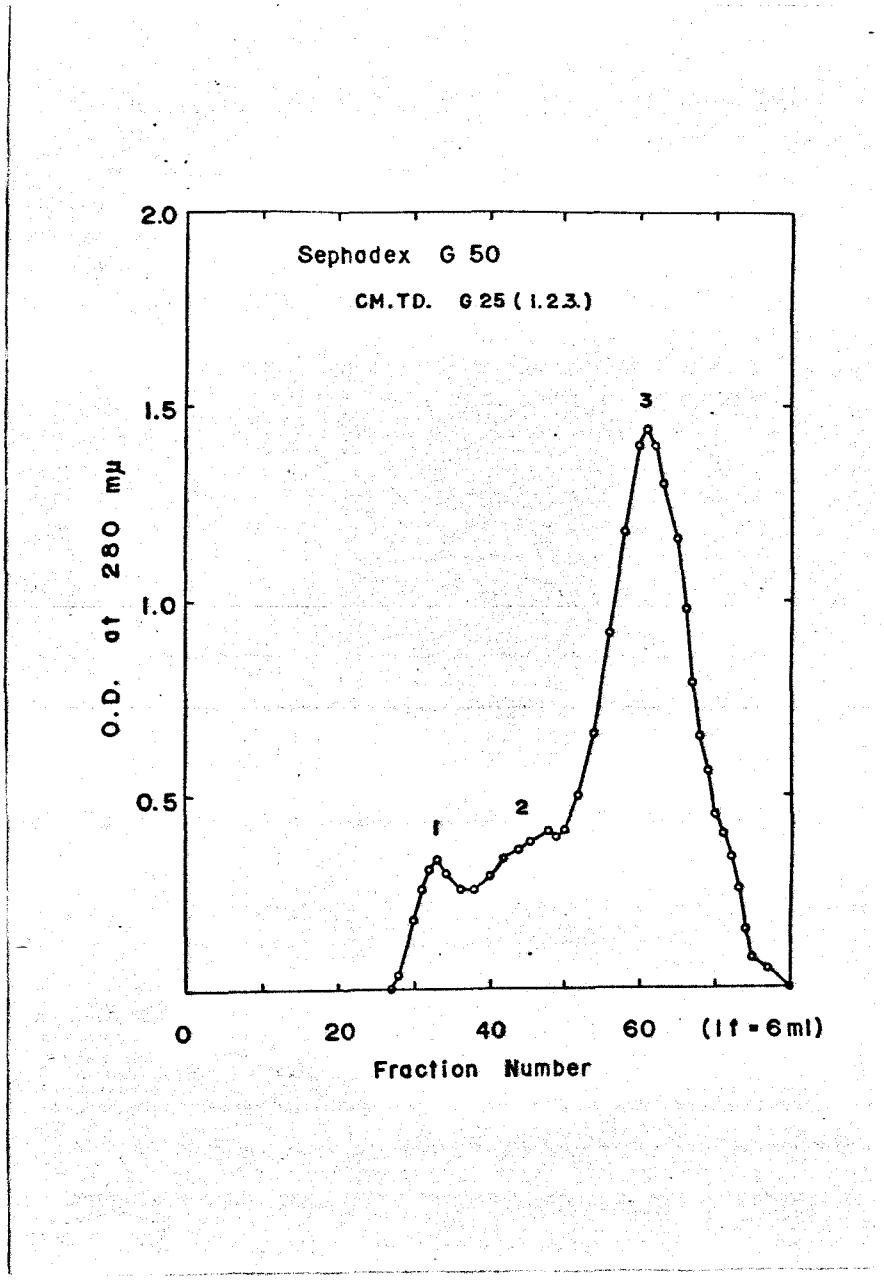



Fig. 11, ( b ). Group Separation of the First Peak, Separated by a Sephadex G 25 Column ( Fig. 11 ( a ) ), by Gel Filtration on a Sephadex G 50 Column ( 3.5 X 70 cm. ).

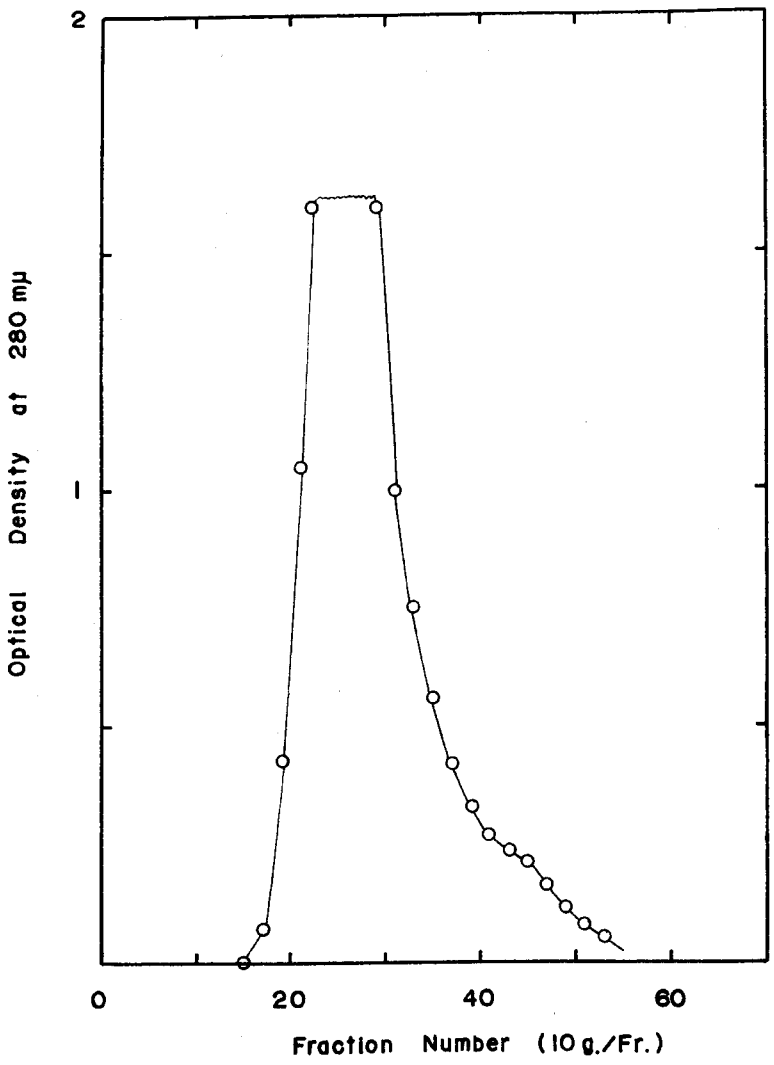



Fig. 12. ( a ). The Pattern of Gel Filtration of R-TF-TD on a Sephadex G 25 Column ( 3 × 30 cm. ).

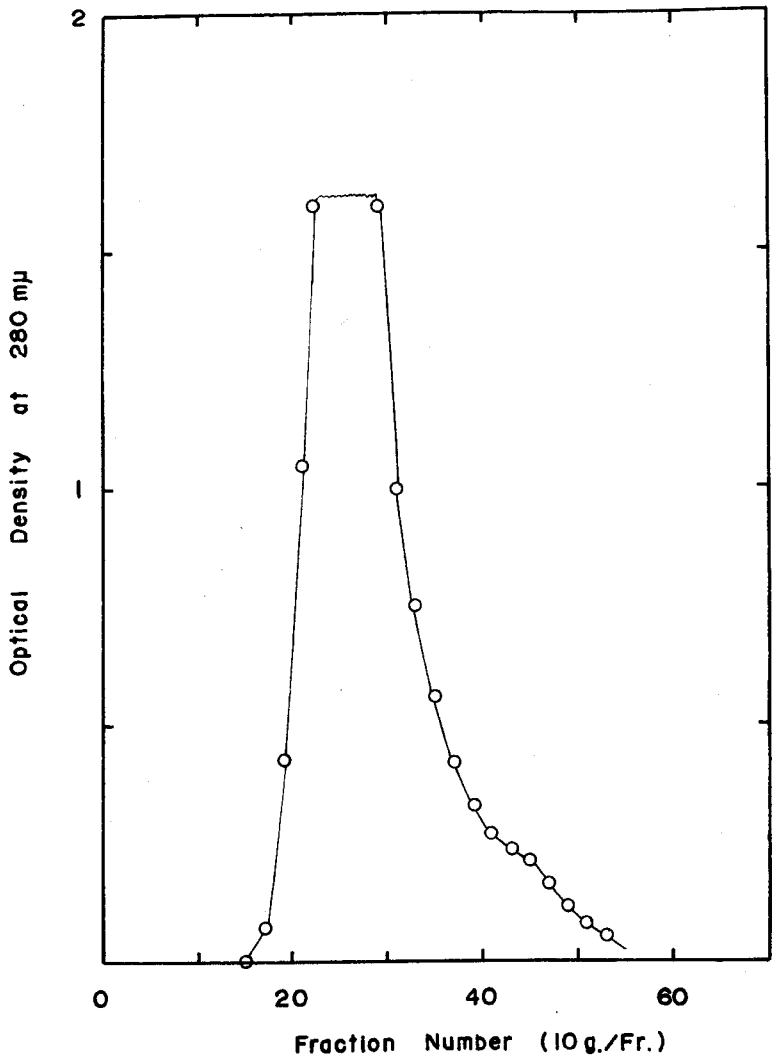



Fig. 12, ( a ). The Pattern of Gel Filtration of R-TF-TD on a Sephadex G 25 Column ( 3 X 30 cm. ).

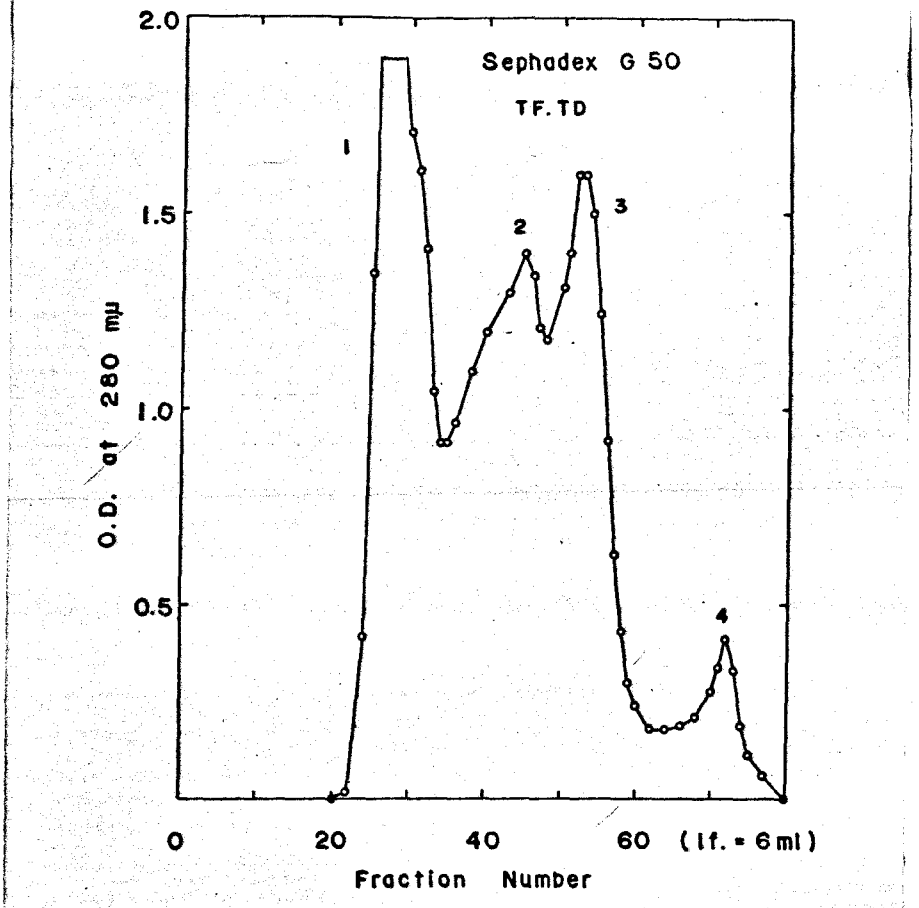



fig. 12 , ( b ). The Pattern of Gel Filtration of R-TF-TD on a Sephadex G 50 Column ( 3.5 X 70 cm. ).

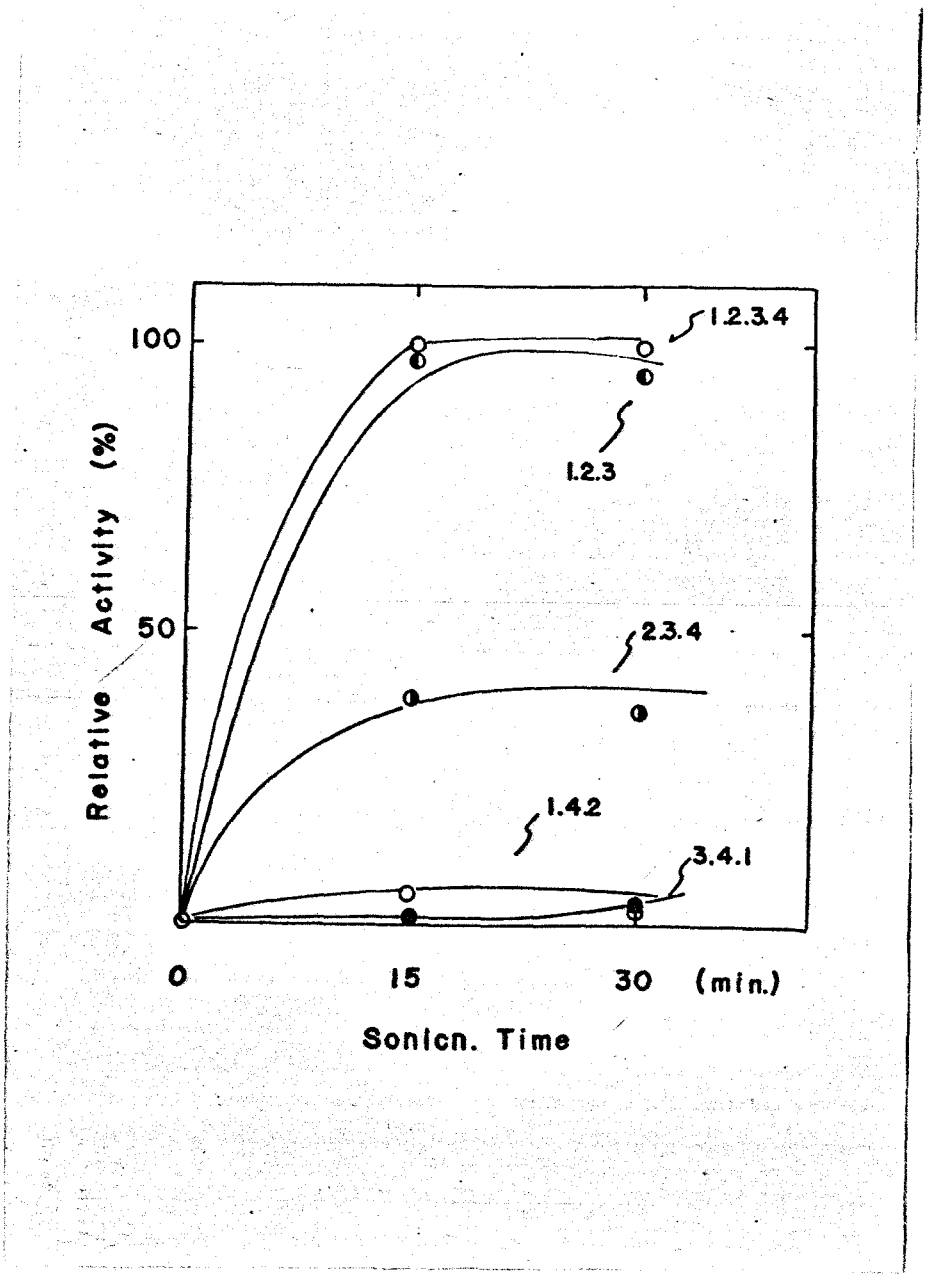



Fig. 13. The Activity-time Curves of Four Combinations of Peptide Groups of R-CM-TD.

Sonic oscillation was performed under the condition of pH 7, $10^{-3}$ M $Ca^{++}$. The concentration was adjusted as the concentration corresponds to 0.2 % R-CM-TD having full components.

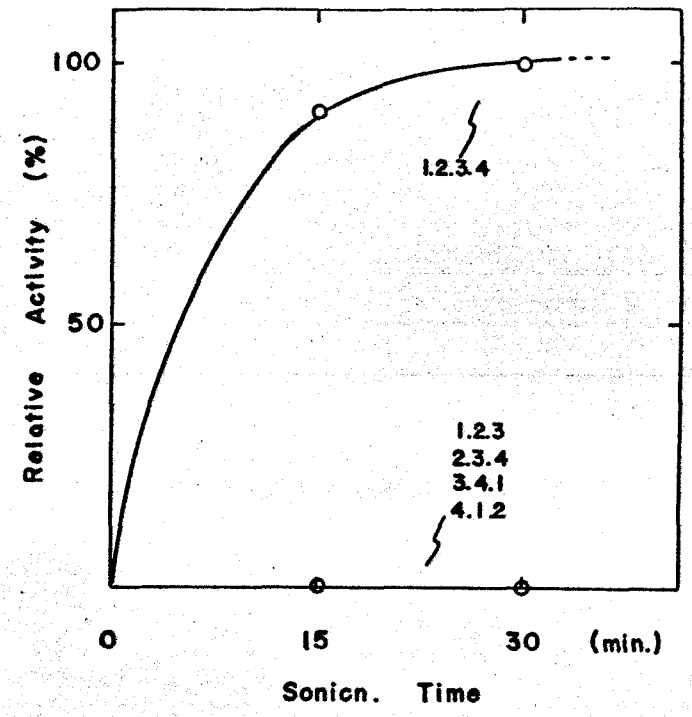
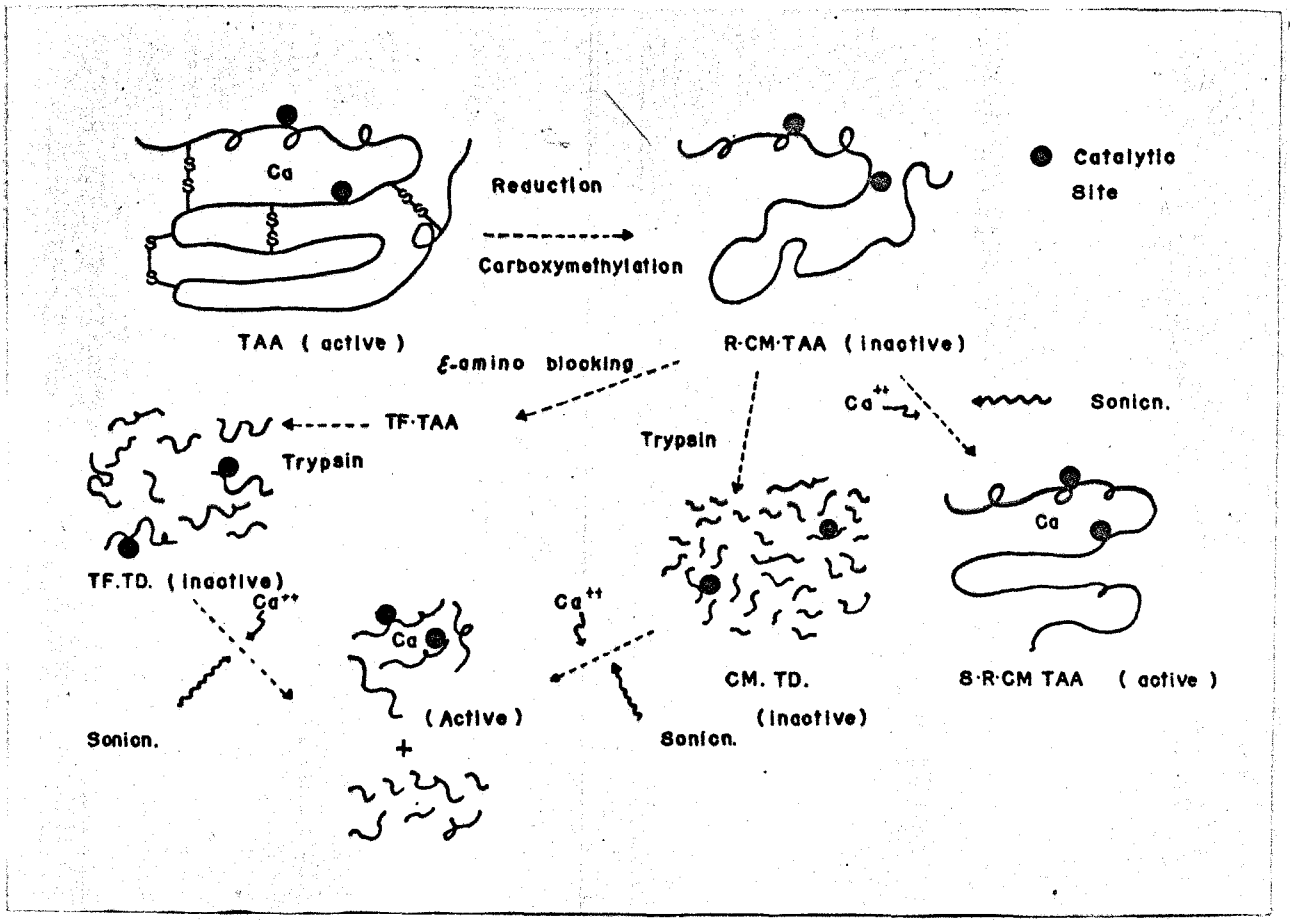



Fig. 14. The Activity-time Curves of Combinations of Peptide Groups of R-TF-TD.

Sonic oscillation was carried out under the conditions of pH 7, $10^{-3}$ M $Ca^{++}$. The concentration of peptide was adjusted at the concentration corresponds to 0.2 % R-TF-TD having full components.




Scheme I. The Schematic Illustration of the Mechanism of Sonic oscillation on R-CM-TAA and Its Derivatives.

Effects of Sonic Oscillation on Taka-Amylase A and Its Derivatives.

# IV. The Some Characters of the Active Derivatives of Reduced Carboxymethylated Taka-Amylase A and of Its Tryptic Digests Produced by Sonic Oscillation.

By Ikunoshin Kato

# Effects of Sonic Oscillation on Taka-Amylase A and Its Derivatives.

## IV. The Some Characters of The Active Derivatives of Reduced Carboxymethylated Taka-Amylase A and of Its Tryptic Digests Produced by Sonic Oscillation.

In the previous paper, it was shown that the reduced-carboxymethylated Taka-amylase A ( R-CM-TAA ), trifluoroacetyl derivative ( R-CM-TF-TAA ) and their tryptic digests revived their amylase activities by the action of sonic oscillation ( 1 ). However, itwas not made clear how the activity revived and what kind of linkages and conformation have formed by sonication.

For the elucidation of the question, some physicochemical experiments concerning the conformation of the revived enzyme derivatives were performed mainly using R-CM-TAA.

The present paper describes the results of optical rotatory dispersion, of the pH effect on reduced viscosity, of difference spectral studies and of the pH stability of active derivatives produced by sonic oscillation. On the basis of the results of the present studies, a probable conformation of the revived protein molecule and the peptide associates by the sonic treatment is proposed.

## EXPERIMENTS AND RESULTS.

Sonicated Proteins.- - - For the physicochemical measurement and pH stability of amylase activity, the following three kinds of proteins were used ; 1.) Sonicated R-CM-TAA. - - - the reduced carboxymethylated Taka-amylase A ( R-CM-TAA ) was subjected to sonication for 30 min. under the optimal conditions as described in the previous paper. 2.) Sonicated R-CM-TD - - - The tryptic digest of R-CM-TAA, ( R-CM-TD ) was sonicated for 30 min. under the same conditions as those used for R-CM-TAA. 3.) Sonicated R-TF-TD - - - The tryptic digests of trifluoroacetylated R-CM-TAA was sonicated under the same conditions as for R-CM-TAA.

Viscosity Measurements.- - - The measurement was performed by using Ostwald type viscometer and the flow time was 60 sec by using deionized water. In the studies of pH effect on viscosity, the pH was adjusted with N NaOH or HCl by Beckman pH meter.

Rotatory Dispersion Studies.- - - Optical rotatory dispersion was measured by Rudolf Precision Polarimeter with Hg lump. Employed sample was 1 % aqueous solution of the sonicated R-CM-TAA and the cell of 10 cm. was used.

The pH Stability of Activity. - - - Samples were exposed on the various pH's of MacIlvain buffer at 45° C and it was pipetted out into M/2 acetate buffer of pH 5.3 at appropriate time interval. Then the solution was kept at a cold room for 24 hours and the assay was performed.

Difference Spectral Studies. - - - Difference spectra were measured by using Shimadzu UV. spectrophotometer with 1 cm. cubet at room temperature.

Amylase Activity.- - - The activity was assayed by measuring its its sacharifing power for amylose ( Nagase ) according to the Fuwa's method ( 2 ).

## EXPERIMENTS AND RESULTS

### Viscosity Studies As a Function of pH.

It is supposed that the conformation changes induced by sonic oscillation will be detected as a viscosity changes. It is expected that if random linear polymer is the products, the pH dependence of viscosity will be observed. On the other hands, no pH dependence will be observed, if the products have rigidly ordered structure. Therefore, the effect of pH on the reduced viscosities of R-CM-TAA and the sonicated R-CM-TAA ( S-R-CM-TAA ) were compared with those of the intact TAA, as shwon in Fig. 1.

- - - - - - - - - - - - - - - - -

Fig. 1. The pH Dependence of Viscosity.

- - - - - - - - - - - - - - - - -

The data show that pH dependence of R-CM-TAA are remarkable compared with that of S-R-CM-TAA of 30 min.' sonication.

The value for S-R-CM-TAA are closely identical with that of the intact TAA.

Optical Rotatory Dispersion.

The results of pH dependence of the reduced viscosity of S-R-CM-TAA suggests that some structural organization were induced by sonic oscillation but it can not be seen what kind of organization occurred. Then, its optical rotatory dispersion of S-R-CM-TAA was studied comparing with that of R-CM-TAA as represented in Fig.2.

- - - - - - - - - - - - - - - - -

Fig. 2. The Optical Rotatory Dispersion Curves.

- - - - - - - - - - - - - - - - -

It waw observed that about 20° of levo rotation was decreased by sonication for 30 min. The values of $\lambda_c$ for R-CM TAA and S-R-CM-TAA were 210 and 245 mμ, respectively. The data shown in Fig. 2. were replotted by Moffitt's plot ( 3 ).

in Fig. 3.

In both cases, R-CM-TAA and S-R-CM-TAA, the slope are close to zero but the height of plot differ from each other. Obviously, these results mean that the both do not have any helical structure.

- - - - - - - - - - - - - - - - - -

Fig. 3. Moffitt's Plot of R-CM-TAA and S-R-CM-TAA.

- - - - - - - - - - - - - - - - -

If some organizations of denatured conformation of R-CM-TAA have induced by sonic oscillation, the difference spectral changes may be expected ( 4 ). Then the differential spectra of TAA, R-CM-TAA and S-R-CM-TAA in acidic solution ( pH 1.8 - 1.9 ) against their neutral solution were measured, as shown in Fig. 4.

As represented in Fig. 4, the blue shift, owing to the denaturation ( 4 ) was observed in both cases, R-CM-TAA and

S-R-CM-TAA, but the definite changes can not be analyzed by the interference of indole chromogen.

- - - - - - - - - - - - - - - - -

Fig. 4. Acidificating Differential Spectra.

- - - - - - - - - - - - - - - - - -

The difference spectra, R-CM-TAA versus S-R-CM-TAA at neutral pH, was also obtained as shown in Fig. 5.

- - - - - - - - - - - - - - - - - -

Fig. 5. The Difference Spectra of R-CM-TAA versus S-R-CM-TAA.

- - - - - - - - - - - - - - - - - -

The data of Fig. 5 indicate the red shift of indole groups but the fine structural analysis is impossible.

Finally, The pH stablities of the revived amylase activities were compared with that of the intact TAA as shown in Fig. 6.

- - - - - - - - - - - - - - - - - - -

Fig. 6. The pH Stabilities of amylase Activities revived by Sonic Oscillation.

- - - - - - - - - - - - - - - - - - -

The results show that revived amylase activities are as stable as the intact TAA in every case and pH dependence is also quite similar to that of the intact TAA.

## DISCUSSION.

Considering the results of viscosity measuerment as shown in Fig. 1, the low value of reduced viscosity of

S-R-CM-TAA suggests that some reorganization of the structure of R-CM-TAA was induced by sonic oscillation. The pH dependence of the reduced viscosity suggests that the sonicated product has the similar hydrodynamic conformation to the groblar intact TAA. If S-R-CM-TAA is a linear polymer, the pH effect should be observed. However the results of viscosity studies do not indicate what kind of ordered structure has reproduced by the sonication.

The results of Optical rotatory dispersion experiments also indicate the possibility that structural reorganization occurred ( Fig. 2 and 3 ). According to the concept of Shelmann et al. ( 5 ), the value of $\lambda_c$ of S-R-CM-TAA indicate the increase of helical contebt by sonic oscillation ,comparing with the value of the original R-CM-TAA.

However the Moffitt's plot, as shown in Fig. 3, showed that $b_o$ was closed to zero but $a_o$ have increased, comparing with those values for the original R-CM-TAA. According to the theory of Moffitt ( 3 ), these results could not precisely be analyzed.

Recently Imahori ( 6 ) proposed a new concept to explain such results of optical rotatory dispersion. He showed that $\gamma$-globuline does not have contain any helical structure

but it could be denatured by denaturating agents and by heating. The Value of $b_o$ does not change and remain constant even after denaturating treatment, while a value changed. He named such structure, as cross β structure.

Taking into consideration his results, the transformation from the structure of R-CM-TAA into that of S-R-CM-TAA is quite similar to the case of γ-globuline. Therefore, it might besupposed that cross β structure was formed by action of sonic oscillation.

Assuming that the sonicated R-CM-TAA has Cross β structure, the pH stability of the revived activities, as shown in in Fig. 6, suggest that the structure of active center of sonicated products are quite similar with of the intact TAA. Then, it leads to a possible consideration that even the structure of active center of the intact TAA are consisted of cross β structure in intrapeptide chain.

## SUMMARY

1.) Viscosity studies were performed by using R-CM-TAA and the sonicated R-CM-TAA. The pH dependence on reduced viscosity of R-CM-TAA was clearly observed but of sonicated R-CM-TAA was not observed like the intact TAA.

2.) Optical rotatory studies show that $\lambda_c$ value of R-CM-TAA and sonicated R-CM-TAA were 210 and 245 mμ, respectively. From the Moffitt's plot, $b_o$ was estimated to be close to zero for both proteins. Then itwas proposed that cross β structure might be formed by sonic oscillation.


## AKNOWLEDGEMENT

Author wish to express his thanks to Prof. K. Narita and Dr. Toda for their kind guidance and valuable discussions through this investigation and also to Sankyo Co., Ltd. for their kind supply of " Takadiastase Sankyo".


## REFERENCES


1.) The third report of this series.

2.) Fuwa, H., J. Biochem., 41, 583 ( 1954 )

3.) Moffitt, W., J. Chem. Phys., 25, 467 ( 1954 )

4.) Tanford,C., and Hauenstein,J.D.,J.Am.Chem.Soc.,78,5287 ( 1956 )

5) Schellmann , C. H.,J. Compt. rend. trav. lab. Carlsberg, Ser. Chem., 30, 463 ( 1958 )

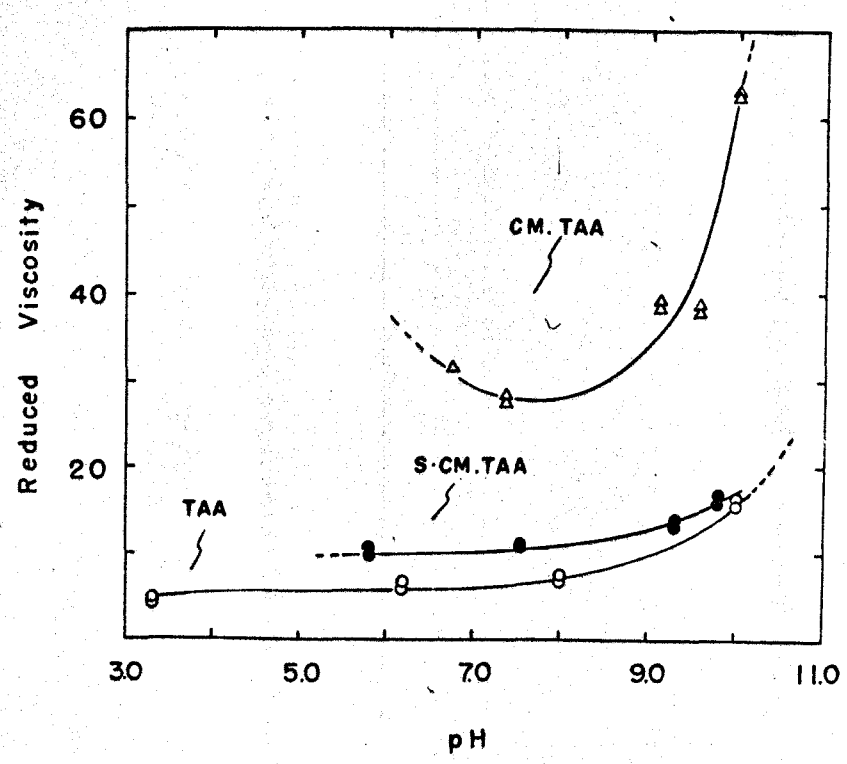



Fig. 1. pH Effect on the Reduced Viscosities.

Viscosity measurement was performed at 30° C by using 30 min. sonicated 1 % aqueous solution. pH was adjusted with NaOH and HCl.

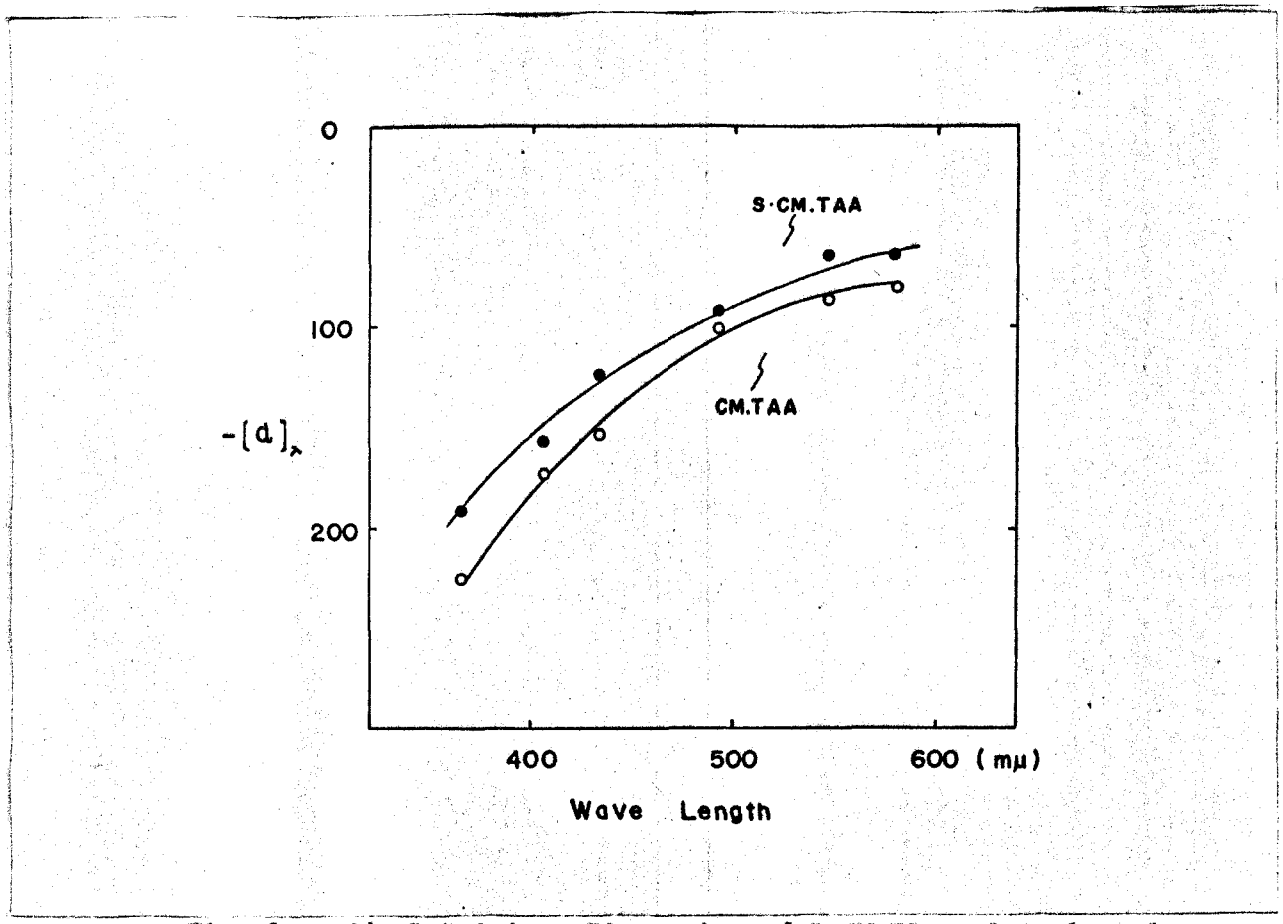



Fig. 2. Optical Rotatory Dispersion of R-CM-TSA and Sonicated R-CM-TAA.

1 % aqueous soln., at room temperature.

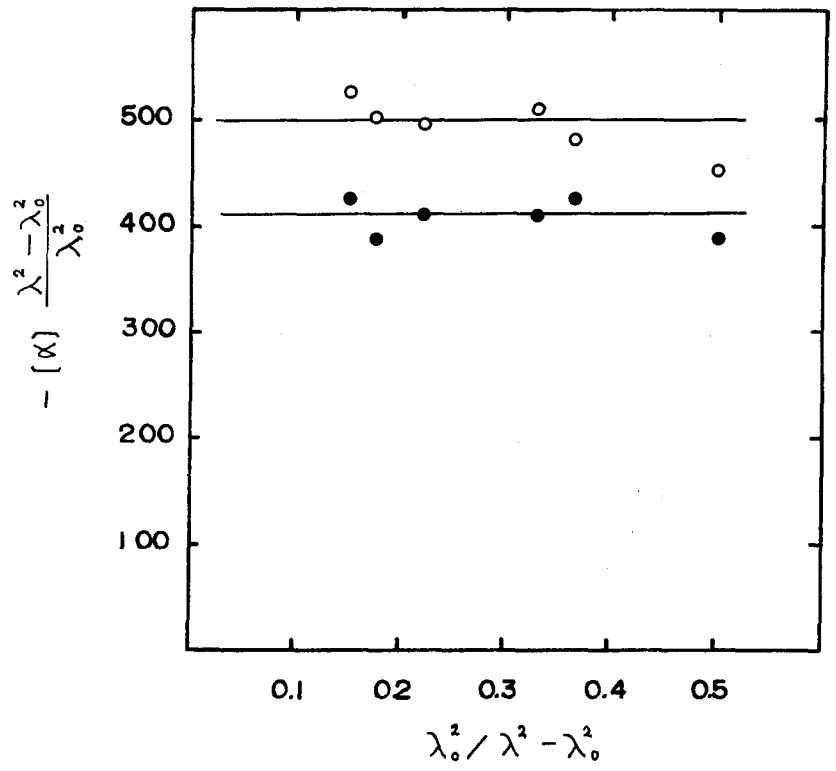



Fig. 3. Moffitt's Plots of R-CM-TAA and S-R-CM-TAA. This plot was obtained from the data as shown in Fig. 2.

—o—, R-CM-TAA and S-R-CM-TAA, —•— .

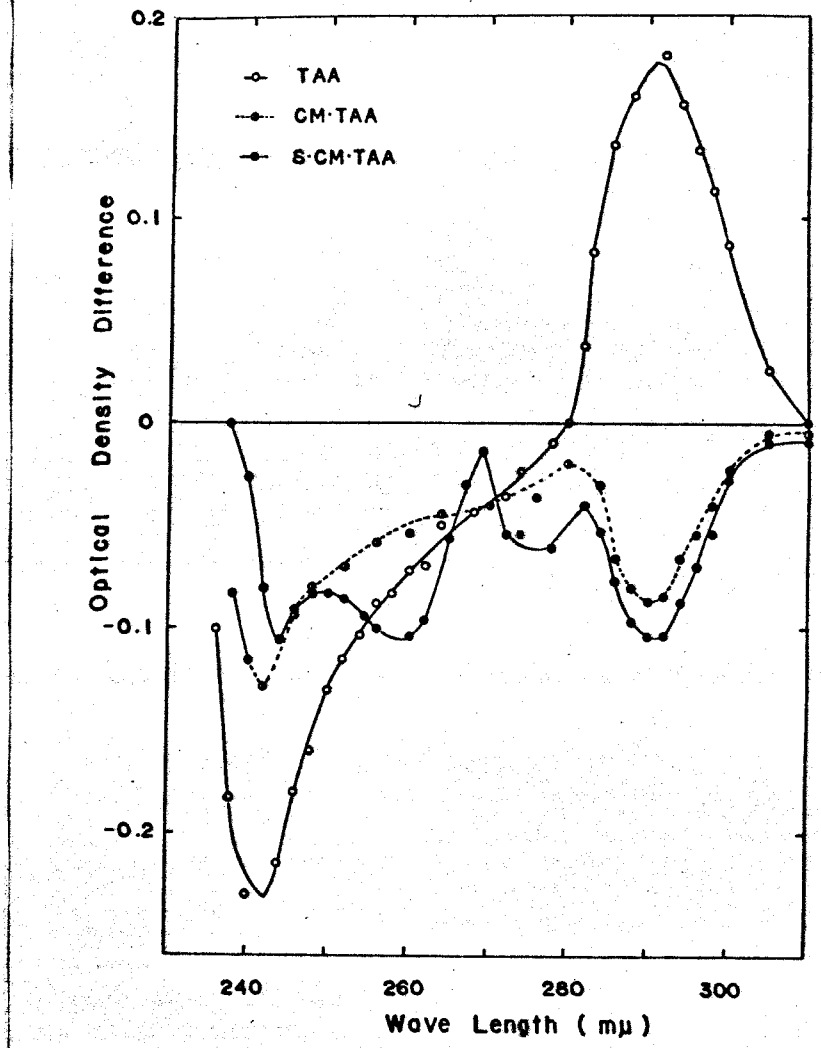



Fig. 4. The Ultraviolet Difference Spectra.

TAA, ref : pH 1.8, sample : pH 6.3

R-CM-TAA, ref : pH 1.9 , sample : pH 7.4

S-R-CM-TAA, ref : pH 1.8 , sample : pH 8.0

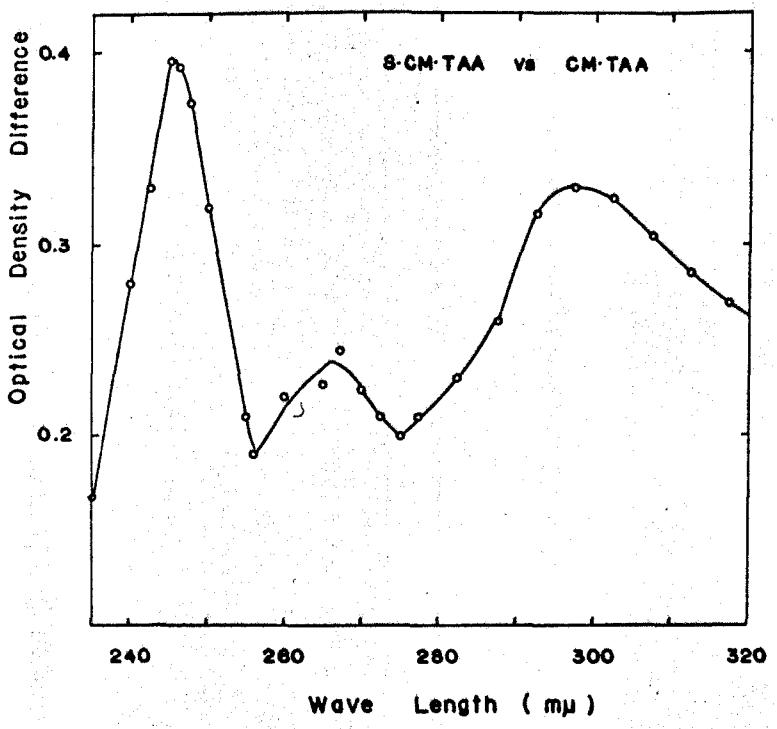



Fig. 5 The Ultraviolet Difference Spectrum of 0.44 % S-CM-TAA versus R-CM-TAA at pH 7.6 at 25° C.

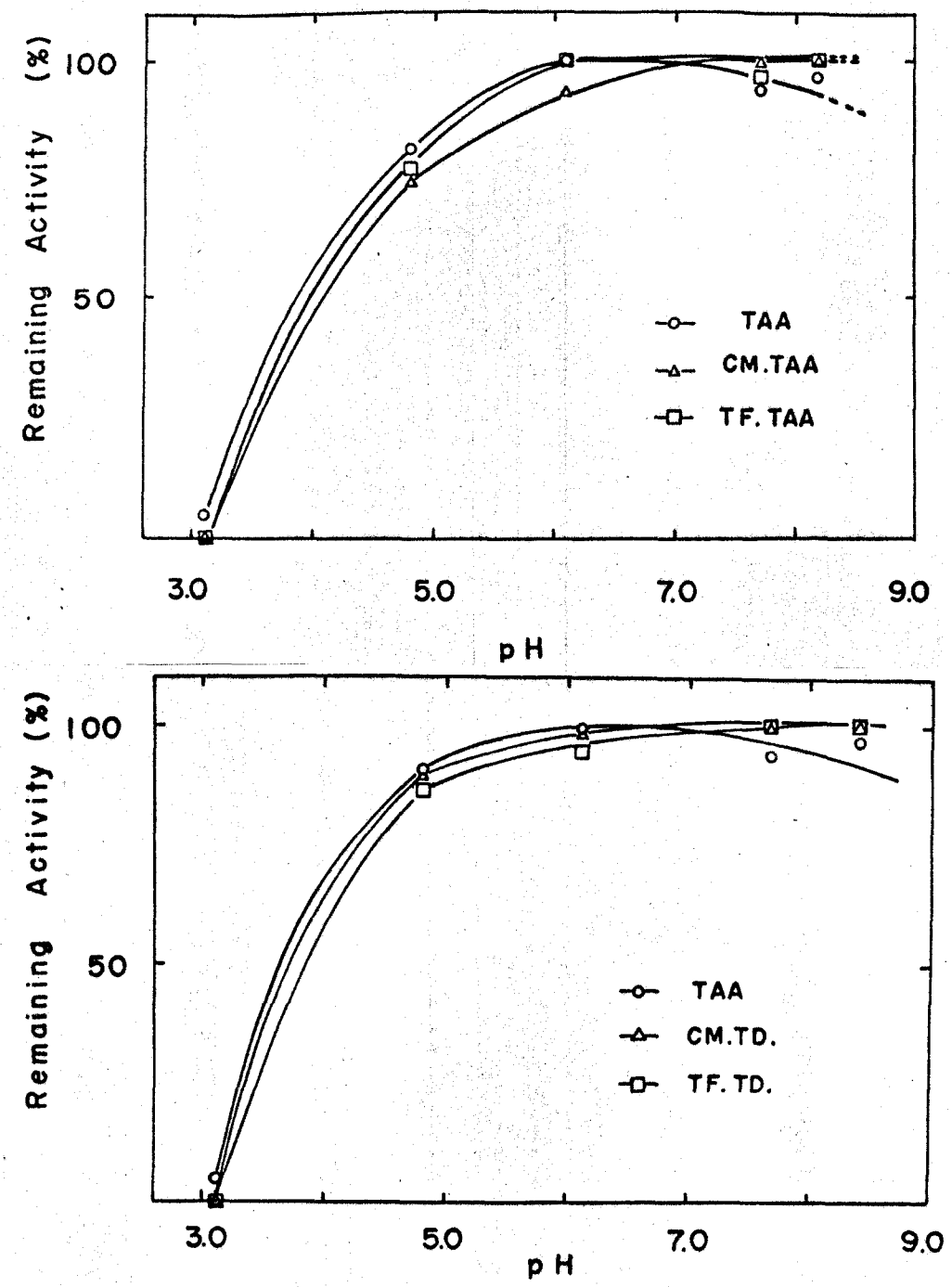



Fig. 6. The pH Stability of the Revived Amylase Activity by Sonic Oscillation.

The Remainning activity at several pH's after 10 min'. exposuring at 45° C were plotted.