CRYSTALLINE PEPSIN

III. Preparation of Active Crystalline Pepsin from Inactive Denatured Pepsin

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INTRODUCTION

The results (1) of the experiments with crystalline pepsin isolated from crude pepsin preparations indicated that the material is a pure substance and that the proteolytic activity is a property of the protein molecule itself and is not due to the presence of a separate non-protein impurity. No indication of the presence of a more highly active non-protein molecule was obtained in the solubility measurements, inactivation experiments, or measurements of the rate of diffusion. In a sense however, this evidence is all negative in that it merely fails to show the presence of a more active molecule. It was shown, for instance, that the loss in activity in alkaline solution was quantitatively paralleled by the production of insoluble denatured protein. It could be objected, however, that the enzyme was liberated from the protein when the latter became denatured and that the enzyme itself was too unstable to exist alone. If it could be shown, however, that reversal of denaturation of the protein was accompanied by reactivation of the enzyme and that the native protein obtained in this way had the same activity as the original protein, strong proof would be furnished for the assumption that the activity was really a property of the protein molecule. In order to account for this result on the hypothesis that the activity was due to the presence of a small amount of a more highly active substance it would be necessary to suppose that the conditions for transforming the denatured protein into the original pro-

tein and for reactivating the enzyme were nearly identical—an assumption which is highly improbable.

It was found by Pawlow and Parastschuk (2) that pepsin solutions which had been inactivated by alkali recovered some of their activity when allowed to stand in nearly neutral solution. It was shown by the writer (1) that this alkali-inactivation of the enzyme is quantitatively paralleled by the formation of denatured protein as determined by the production of protein insoluble near the isoelectric point. The procedure used by Pawlow and Parastschuk for reactivating the enzyme was identical with that found by Anson and Mirsky (3) for the reversal of denaturation of proteins. This suggests that the reactivation of pepsin is due to the reversal of coagulation of the pepsin protein. If this explanation is correct it should be possible to isolate active pepsin protein from such reactivated solutions. The experiments reported in this paper show that this can be done.

Experimental Results

Pawlow and Parastschuk's results were repeated more carefully by Tichomirow (4) who apparently could recover about 10 per cent of the original activity using dog gastric juice. Tichomirow inactivated the enzyme by adding strong alkali or sodium carbonate and then titrated the solution to slightly less than neutrality as shown by litmus. When the solution was allowed to stand under these conditions a gradual increase in activity was found. Exact repetition of these experiments is difficult since at the time of Tichomirow's work pH measurements were not known. The exact degree of neutralization could not be duplicated with certainty from the data given. It is evident from Tichomirow's work, however, that the reaction is dependent upon the pH at which inactivation takes place, the pH and temperature at which reactivation occurs, and the length of time for which the solutions are allowed to stand under these conditions. In addition, the salt concentration and enzyme concentration are other possible variables.

It has been shown by Goulding, Borsook and Wasteneys (5) that two reactions are involved in the inactivation of pepsin by alkali. The first reaction is instantaneous and its *extent* is determined by the pH to which the solution is brought, while the *rate* of the second reaction

is determined by the pH. When the percentage inactivation caused by the instantaneous reaction is plotted against the pH a titration curve is obtained with a pK of about 6.9. This result has been confirmed with crystalline pepsin solutions, and it was shown in addition that the percentage inactivation was exactly parallel with the percentage of the protein denatured.

Preliminary experiments showed that a solution of crystalline pepsin which has been inactivated by titrating with NaOH to pH 10 or 11 (pink to nile blue) becomes slightly active again when allowed to stand

TABLE I

Effect of pH of Solution during Reactivation

100 ml. crystalline pepsin solution titrated to pH 10.5 with NaOH at 22°C. for 10 min. Titrated to pH 6.0 + HCl and diluted 1/8 with M/100 citrate buffer of different pH. Allowed to stand at 22° C. for 18 hrs. and activity determined.

pH of solution after diluting with citrate buffer		5.0	5.3	5.6	5.9	6.2
$[PU]_{ml.} \times 10^3$ of original solution	gelatin V	1.0	1.0	1.0	1.0	1.0
	casein F	100	100	100	100	100
After inactivation	gelatin V	0	0	0	0	0
	casein F	0	0	0	0	0
After reactivation	gelatin V	0.02	0.07	0.13	0.16	0.12
	casein F	0.10	0.40	0.40	0.20	0.10
Per cent of original, reactivated	gelatin V	2.0	7.0	13.0	16.0	12.0
	casein F	0.10	0.40	0.40	0.20	0.10

after partial neutralization. The yields, however, were less than 1 per cent instead of 10 per cent as described by Tichomirow. The conditions were therefore varied systematically in an attempt to increase the yield. The first condition studied was the effect of varying the pH to which the solution was brought after complete inactivation by alkali. It was found that the maximum reactivation was obtained at about pH 5.4 for the casein-hydrolyzing activity, while the gelatin liquefying activity was recovered more completely

in slightly more alkaline solution.[1] The results of one of these experiments are shown in Table I.

In the experiment just described inactivation was caused by strong alkali and was therefore probably due almost entirely to the instantaneous reaction rather than to the slower secondary reaction. It seemed possible that better yields might be obtained if the inactivation were brought about by the slow reaction. However, experiment showed that this was not the case but that on the contrary no reactivation could be obtained in solutions which had been allowed to inacti-

TABLE II

Effect of pH during Inactivation on Reactivation

Series of 10 ml. crystalline pepsin solutions titrated to pH noted. Kept at 20°C. until more than 90 per cent of activity had been lost. Titrated to pH 5.4, kept at 22°C., and activity determined after 24 to 48 hrs.

pH at which inactivation occurred		7.0	8.0	8.5
$[PU]_{ml.} \times 10^3$ of original solution	gelatin V.	4.0	4.0	4.0
	casein F.	400	400	400
After inactivation	gelatin V.	0.4	0.5	0.1
	casein F	0.10	0	0
After reactivation	gelatin V.	0.4	0.6	0.2
	casein F	0.8	0.4	0.35
Per cent reactivated	gelatin V	0	2.5	2.5
	casein F	0	0.1	0.1

vate slowly at lower pH's. The results of this experiment are shown in Table II.

Effect of the Time of Standing after Neutralization.—When the solution is brought back to pH 5.4 and tested at once for activity it is found to be completely inactive. The activity slowly increases for

[1] The variation in the activity as measured by the liquefaction of gelatin or the hydrolysis of casein after alkali inactivation is due to the fact that the crude pepsin contains a gelatin liquefying enzyme which can only be removed by repeated crystallization. This gelatin splitting enzyme is more resistant to alkali than the pure pepsin. This point will be discussed more fully in a subsequent paper.

24 to 48 hours and then decreases so that apparently there are again two reactions, one of which leads to the formation of active enzymes while on the other hand, the active material is being slowly destroyed. The amount of active material actually recovered then is the result of these two reactions. The results of such an experiment are shown in Table III.

Effect of Concentration on Percentage of Reactivation.—The work of Anson and Mirsky on the reversal of denaturation of proteins indicates that the reaction is dependent somewhat upon the solubility of the denatured protein. Conditions which increase the solubility of the denatured protein are favorable for reversal while conditions which

TABLE III

Effect of Time at pH 5.4 on Reactivation

0.05 per cent pepsin solution titrated to pH 10.5 with NaOH at 22°C. for 10 min. Titrated to pH 5.4 with N/2 HCl. Activity determined after various time intervals at 22°C.

Hrs. at 22°C.	$[PU]_{ml.} \times 10^3$	
	Gelatin V.	Casein F.
0	0	0
2		0.02
18	0.03	0.20
42	0.03	0.21
65	0.04	0.25
90	0.045	0.12
114	0.040	0.10

cause precipitation of the denatured protein are unfavorable. It seemed possible therefore that better yields would be obtained with more dilute solutions and this was found to be the case. There is even some indication that the concentration of active material recovered is nearly constant so that the percentage of activity recovered increases with the dilution. Table IV shows the results of the experiment in which the solution was diluted to various extents after being titrated to pH 5.4. Practically, however, it is not possible to work with solutions containing much less than 1 mg. of nitrogen per ml. owing to the very large volume.

A number of other experiments were done in an attempt to increase the percentage yield but without success. It is quite possible that the difference between these results and those of Tichimorow are due to the difference between dog pepsin and pepsin from pigs, since Anson and Mirsky have found that there is very considerable difference in the ease of reversal of hemoglobin from different animals.

Tests for the Presence of Inhibiting Substances.—The possibility exists that the loss in activity is due not to destruction of the enzyme but to the formation of an inhibiting substance which depresses the activity of the enzyme and which disappears on standing at pH 5.4 and thus liberates the active enzyme. This possibility can be rendered remote by testing the effect of the inactivated solution upon the ac-

TABLE IV

Effect of Concentration on Reactivation

Solution of crystalline pepsin titrated to pH 10.5 with N/2 NaOH at 20°C. for 10 min. Titrated to pH 5.4 with HCl, diluted with water, and kept at 22°C. for 18 hrs.

Mg. N/ml.	8	4	2	1	0.5
$[PU]_{ml.} \times 10^3$ (casein F.) — original solution	800	400	200	100	50
Immediately after titrating to pH 5.4	0	0	0	0	0
After 24 hrs. at pH 5.4	1	1	0.7	0.5	0.4
Per cent of activity recovered	0.1	0.25	0.35	0.5	0.8

tivity of normal pepsin solutions. If an inhibiting substance were present it would be expected that the activity of a known amount of active enzyme when added to this inactivated solution would be decreased since a large excess of inhibiting agent is usually necessary to completely inactivate the enzyme. No evidence for the presence of inhibiting substances could be found since active pepsin solutions diluted with a large excess of inactive solution show the same activity as when diluted with water. This result is shown in Table V.

Tests for Completeness of Inactivation.—As shown in the previous paper the denatured protein itself is rapidly digested by the enzyme, and this furnishes a very sensitive test for the presence of any active

pepsin in the solution after inactivation by alkali. The inactivated solution when brought to pH 2.5 to 3 forms a heavy precipitate of denatured protein. Under these conditions this protein is rapidly digested if any active enzyme is present. It was found that when the inactivated solution was titrated to pH 2.5 no increase in soluble nitrogen could be detected after 24 or 48 hours at 37°C. The addition of 4 per cent of the same solution which has been allowed to reactivate at pH 5.4 caused a marked increase in the amount of soluble nitrogen under these conditions (Table V). It may therefore be stated with

TABLE V

Activity Determination on Inactivated Pepsin Solution

6 per cent solution of crystalline pepsin titrated to pH 11.0 with N/2 NaOH

	$[PU]_{ml.} \times 10^3$	
	Gelatin V.	Casein F.
50 ml. titrated to pH 5 rapidly, slightly cloudy solution = a.....	0	0
100 ml. titrated to pH 3 rapidly, precipitate forms = b.....	0	0
25 ml. titrated to pH 5.4 and kept at 22°C. for 24 hrs. = c.....	0.05	0.5
Active pepsin solution diluted 1/100 with H_2O.....	1.5	150
with Solution a.....	1.6	165

0, 1 and 2 ml. Solution c added to 3 portions of 25 ml. of Solution b and nitrogen determined per ml. filtrate after 0 and 24 hrs. at 37°C.

Ml. reactivated Solution c.....	0	1	2
Mg. N/ml. filtrate after 0 hrs. at 37°C.....	0.10	0.12	0.15
after 24 hrs. at 37°C.....	0.10	0.20	0.25

considerable certainty that not more than 2 per cent of the activity present in the reactivated solution was present after inactivation due to incomplete destruction of the original activity. In this experiment also no filtration or separation was made so that complicating factors such as adsorption of the active material by the precipitate, etc. seemed to be ruled out.

It had been found before that a mixture of active and inactivated pepsin could be separated by precipitating the inactive and denatured protein with a mixture of sulfuric acid and sodium sulfate. In the

reactivated solutions obtained in the present work, however, it was found that the very small amount of active material present was carried down nearly completely by the denatured protein under these conditions, so that a new method of separation had to be worked out. After a number of failures it was found that most of the denatured protein could be precipitated from the solution by adding acid very slowly and carefully to the reactivated solution until it was just possible to filter off the precipitate formed. Under these conditions the active material is not carried down by the precipitate but remains in the filtrate. The addition of a slight excess of acid, however, causes all the active material to be retained by the precipitate. It seems probable that this result is due to the charge on the precipitate. The active pepsin is isoelectric at about pH 2.7 while the denatured protein is apparently isoelectric somewhere around 4.5, so that between these two pH's the two proteins are oppositely charged and precipitate each other, while if the reaction is adjusted so as to be on the alkaline side of the isoelectric points of both proteins they may be separated. The gelatin liquefying material is carried down with the precipitate even under these conditions so that this step serves to separate the reactivated pepsin from the "gelatinase" as well as from the denatured pepsin. The active pepsin may be recovered from this filtrate by adjusting to pH 3 and half saturating with magnesium sulfate. The precipitate obtained in this way contains about 2/3 of the total recovered activity but has a specific activity of only about 1/10 that of the original crystalline pepsin and evidently still contains large amounts of the denatured protein. Most of the latter may be removed by dialysis against 1/100 normal hydrochloric acid for 2 or 3 days. Under these conditions the denatured protein is partly digested by the active enzyme present. The resulting clear solution is precipitated with magnesium sulfate and the precipitate now has about half of its original specific activity. It still contains quite a large amount of some protein material which appears to be isoelectric at about pH 5 and from which the active enzyme can be separated only with great difficulty. Successive fractionation with magnesium sulfate at pH 5 gradually removes this inactive protein but no sharp separation could be made, and there is considerable loss in material at this point. Eventually, however, it was possible to obtain a small amount of a

TABLE VI

Inactivation and Reactivation of Pepsin and Isolation of the Reactivated Enzyme

Procedure	Fraction No.	Total [PU]		[PU]gm. N	
		Gel. V.	Cas. F.	Gel. V.	Cas. F.
100 gm. crystalline pepsin dissolved in 1 l. H_2O		17	1700	1	100
Titrate to pH 10.5 (pink to nile blue) + N/2 NaOH (800 ml.) at 22°C. for 10 min.		<0.01	<1	0	0
Dilute to 10 l.					
Titrate to pH 5.4 with N/5 HCl, stirred in slowly so as to avoid local precipitation, at 22°C. for 24 hrs.	1	1.0	15	0.06	1.0
Titrate with N/5 HCl slowly until flocculent precipitate just forms (pH about 5.2).					
Filter.					
Filtrate	2	0.12	10		4
Filtrate 2. Titrate to pH 3.0 + H_2SO_4, $MgSO_4$ added till flocculent precipitate forms (about 350 gm. $MgSO_4$ per l.). Allowed to stand at 6°C. for 48 hrs. Supernatant siphoned off and precipitate concentrated by centrifuging. Centrifuged cake put in collodion sacs and dialyzed against 0.01 HCl at 22°C. for 48 to 60 hrs. Nearly clear solution. Centrifuge. Supernatant titrated to pH 5.4 with NaOH and $MgSO_4$ added until precipitate forms.					
Centrifuge, precipitate inactive.					
Supernatant	3	0.012	1	0.6	40
Supernatant 3. $MgSO_4$ added until flocculent precipitate forms.					
Centrifuge, precipitate weak activity. $MgSO_4$ added again to supernatant until precipitate forms. This process is continued until supernatant fluid has about proper specific activity per gm. N. The combined precipitates may be fractionated again and some active material recovered.					
Combined Mother Liquors from $MgSO_4$ Precipitate.	4	0.006	0.5	0.9–10	90–100
Solution 4. Titrate to pH 3.0 with H_2SO_4. Filter if cloudy and precipitate discarded.					
Filtrate. Saturate with $MgSO_4$ and filter.					
Precipitate	5	0.002	0.17	0.9–10	90–100
Precipitate 5 from 3 preparations combined. Dissolve at 40°C. with 0.2 to 0.3 ml. of water and 0.1 ml. N/10 Na acetate. Cool slowly. Typical hexagonal bipyramids in clusters form after 2 to 3 hrs.					

protein which had the general properties of the original crystalline pepsin. A summary of the method of isolation is shown in Table VI. About 2 kg. of the crystalline pepsin were used and the yields combined.

Control experiments like those shown in Table V were made on each preparation and showed that not more than 2 per cent of the activity could have been due to the presence of original enzyme which had not been inactivated. A small amount of this reactivated protein was crystallized under the same conditions as were used in crystallizing the original pepsin. It formed typical hexagonal bipyramids indistinguishable from those of the original pepsin. The activity as

TABLE VII

Comparison of Properties of Original and Reactivated Pepsin

	$[PU]_{gm.\ N}$			Mg. N to coagulate 5 ml. milk at 35°C. in 2 hrs.	$[\alpha]^{D}_{22°C.}$ pH 4.6
	Gelatin V.	Casein F.	Edestin F.		
Original crystalline pepsin	1.1	92	98	<0.001>0.0005	−70
Reactivated pepsin	1.0	95	100	<0.001>0.0005	−100 ± 10
	0.8	98	110		

measured by the liquefaction of gelatin, the hydrolysis of casein, the hydrolysis of edestin and the coagulation of milk was the same as that of the original pepsin.[2] The optical rotation was measured on a very dilute solution and apparently showed a slightly different value from that of the original pepsin. The reading was somewhat uncertain, however, and it is doubtful whether this difference is significant. The

[2] It will be noted that the activity of the recovered as well as the original pepsin as determined by the digestion of edestin is only half of that originally reported for crystalline pepsin. This difference is apparently due to some difference in the edestin preparation since a different preparation was used in the present work. Unfortunately the edestin preparation used previously had been entirely used up but a sample of the original pepsin preparation was at hand both dry and in glycerin solution. This preparation now shows the same activity in all other respects as that found earlier but is apparently less active with the new edestin.

protein is denatured and the activity lost again in alkaline solution. The results of these determinations are shown in Table VII.

Attempts were made to compare the solubility of the reactivated and original pepsin but owing to lack of material these were not conclusive. It was found that the active material forms solid solutions with the protein which is carried along in the purification and which is apparently isoelectric at about pH 5. Addition of this protein to pure pepsin caused a marked decrease in solubility. The results with the rennet action confirmed Pawlow and Parastschuk's conclusion that the apparent separation of rennet from casein by alkaline activation is really due to the fact that the pepsin is reactivated under the conditions used for the coagulation of milk but not under the conditions used for the digestion of casein.

Experimental Methods

The methods used were the same as those described in the study of crystalline pepsin. The pH determinations were made colorimetrically without correcting for any effect of the protein present and are only approximately correct.

SUMMARY

1. Pepsin solutions which have been completely denatured and inactivated by adjusting to pH 10.5 recover some of their activity when titrated to about pH 5.4 and allowed to stand at 22°C. for 24 to 48 hours.

2. Control experiments show that this inactivation and reactivation are probably not due to the effect of any inhibiting substance.

3. A method of isolation of the reactivated material has been worked out.

4. The reactivated material recovered in this way is a protein with the same general solubility, the same crystalline form, and the same specific proteolytic activity as the original crystalline pepsin.

5. This furnishes additional proof that the proteolytic activity is a property of the protein molecule.

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