Smith[13] has drawn attention to the seeming existence of a relationship between the acid strength of the acyl group and the rate of hydrolysis of the acyl amino acid derivative by carboxypeptidase. It was anticipated that the greater the acid strength of the acyl group, the greater would be the rate of hydrolysis as is known to be the case in acid–base hydrolysis. This relationship does have considerable validity; however, trichloroacetic acid is a much stronger acid than chloroacetic acid, which would lead one to expect a greater rate of hydrolysis for the trichloroacetylated amino acids. The results obtained in the present study do not support this concept, in fact, they contradict the notion and demonstrate that something more than acid strength is involved in the hydrolytic process.

The greater activity possessed by hippuryl-DL-leucine and probably hippuryl-DL-norleucine as compared to hippuryl-L-tyrosine was utterly unexpected. As a possible explanation, the question of the presence of a substrate inhibition, dependent upon initial substrate concentration, as regards the tyrosine derivative may be raised in view of the appearance of this phenomenon with chloroacetyltyrosine and with other aromatic amino acid derivatives.[11]

It is noteworthy that similar relative positions were maintained by the hippuryl and chloroacetyl derivatives of leucine, norleucine, methionine and valine. This relative order was also supported from observations on the trichloroacetyl series.

With the exception of the tyrosine compounds and the isoleucine–norleucine relationships, the relative positions between the leucine compounds (standards for the proteolytic quotients) and the other derivatives were, as was expected, reproduced in each series. Further, the hippuryl derivatives appear to be the most potent synthetic substrates for carboxypeptidase thus far produced.

p-Nitrocarbobenzoxyglycyl-L-leucine.—A proteolytic coefficient of 2.0 was obtained for this compound. This indicates that it is only slightly poorer a substrate than carbobenzoxyglycyl-L-leucine (Table I). Though a nitro group in the para position would tend to prevent the secondary peptide nitrogen atom from entering an unfruitful ring A (such as between N_1 and N_2, I), the nitro group itself is a large group with a high dipole moment which could present steric and electrostatic interference to the formation of the proper ES complex.

Acknowledgment.—The author wishes to express his gratitude to Dr. David M. Greenberg for his support of this work.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

Additional Studies of the Properties of Taka Amylase[1]

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A study has been made of the stability and certain other properties of highly purified maltase-free and of crystalline maltase-free taka amylase. Calcium ions do not appear to activate taka amylase but do protect it from inactivation under unfavorable conditions. These and other properties of taka amylase are presented and discussed.

Introduction

Quantitative observations of the properties of highly purified or crystalline enzymes are needed to increase our understanding of their nature and of the mechanism of their action and to define the conditions for additional studies. The work reported here deals with such observations for highly purified and for crystalline maltase-free taka amylase.

Experimental

Amylase.—Most of the work reported here was carried out with highly purified maltase-free taka amylase.[3,4] Essential points were repeated with crystalline taka amylase, prepared in general as reported by Fischer and de Montmollin.[5]

Results and Discussion

General Properties of Taka Amylase.—Both types of amylase solution gave essentially the same results. Solutions of the highly purified maltase-free but uncrystallized preparations had the same saccharogenic and amyloclastic activities[6] as solutions of the crystalline amylase.[7] In both cases, the amylase produced 2400 times its weight of maltose equivalents in 30 minutes at 40° from 1% Lintner's soluble potato starch adjusted to 0.01 *M* acetate and *p*H 5.0.[6] In both cases, the solutions were maltase-free and the uncrystallized preparations were found by selective inactivation measurements[3,4,8] to be free from extraneous dextrinase and other contaminating glucosidase activities. Data for this statement are given later.

Several preparations of highly purified maltase-free taka amylase with constant maximum amylase activity were found to be at least 90% homogeneous proteins by electrophoretic measurements.[9] The

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(2) The data reported here are taken, in part, from a dissertation submitted by Virginia M. Hanrahan in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry under the Faculty of Pure Science of Columbia University.

(3) M. L. Caldwell, R. M. Chester, A. H. Doebbeling and G. W. Volz, *J. Biol. Chem.*, **161**, 361 (1945).

(4) Virginia M. Hanrahan, Dissertation, Columbia University, New York, N. Y., 1950.

(5) E. H. Fischer and R. de Montmollin, *Helv. Chim. Acta*, **34**, 1987 (1951).

(6) M. L. Caldwell and S. E. Doebbeling, THIS JOURNAL, **59**, 1835 (1937).

(7) V. M. Hanrahan and M. L. Caldwell, *ibid.*, **75**, 2191 (1953).

(8) R. B. Alfin and M. L. Caldwell, *ibid.*, **70**, 2534 (1948).

(9) The electrophoretic and sedimentation measurements were carried out by Dr. Maxine McKenzie in the Laboratory of Professor Dan Moore.

electrophoretic analyses were carried out at *p*H 5.0, 6.0 and 7.4 for periods of time up to 3 hours. Figure 1 shows the ascending and the descending boundaries obtained with one of the preparations after 49 minutes in acetate buffer at *p*H 6.0. Similar diagrams were obtained with each of the other preparations at each of the *p*H values and time periods studied. The electrophoretic mobility of the protein in acetate buffer at *p*H 6.0 and at an ionic strength of 0.2 was -4.9×10^{-5} cm.^{2}/second/volt. This value is in the same range as that reported by Fischer and de Montmollin[10] for their crystalline taka amylase. Before being subjected to electrophoresis, the amylase solutions in distilled water were dialyzed at 0° to equilibrium against the buffer to be used. No change was found in the amylase activity of the solutions after the dialyses.

Sedimentation measurements[9] with the same preparations of taka amylase indicated homogeneity of the protein. The sedimentation constant of taka amylase is 4.3×10^{-13} seconds. The absorption spectrum of solutions of taka amylase in distilled water gave an absorption maximum at 2800 Å., typical of proteins. A similar absorption curve was obtained by Fischer and de Montmollin with their crystalline taka amylase[10].

Ultimate Analysis.—Upon ultimate analysis,[11] highly purified maltase-free taka amylase preparations gave: carbon, 47.22%; hydrogen, 7.05%; nitrogen, 12.08%; phosphorus, 0.00%; sulfur, 1.11% and ash, 1.93%. Fischer and de Montmollin[10] report nitrogen, 12.9%, and phosphorus, 0.03%, for their crystalline taka amylase. In both cases, the percentage of nitrogen is low for a pure "simple" protein. This low percentage of nitrogen may be accounted for by the fact that taka amylase appears to contain or to be contaminated with traces of carbohydrate. Thus, aqueous solutions of highly purified and also of recrystallized maltase-free taka amylase containing 8 mg. of amylase preparation or crystals per ml. gave positive Molisch reactions as well as the usual protein color reactions. The nature of this carbohydrate has not yet been established.

Emission Spectrographic Analyses.—Emission spectrographic analyses[12] of recrystallized taka amylase indicated the presence of traces of calcium and of copper and possible traces of magnesium. The concentration of calcium was estimated to be between 0.03 and 0.05 mg. of calcium per 100 mg. of the protein. Sensitive lines were barely visible for iron and not visible for aluminum.

Solubility.—Taka amylase is relatively soluble in water.[10] A solution of crystalline taka amylase with 142 mg. per ml. is obtained readily in distilled water at 0°. In the presence of 0.25 saturated ammonium sulfate, a concentration of 173 mg. per ml. of crystalline taka amylase has been obtained at 0°. The solubility of taka amylase in aqueous solutions in the presence of ammonium sulfate is higher at lower temperatures and decreases as the temperature is increased. Less ammonium sulfate is required to salt taka amylase out of its aqueous solutions at room temperature than at 0° to 10°. This influence of temperature is reversible. The precipitate formed by the addition of ammonium sulfate to an aqueous solution of taka amylase at 20° will dissolve at 0°.

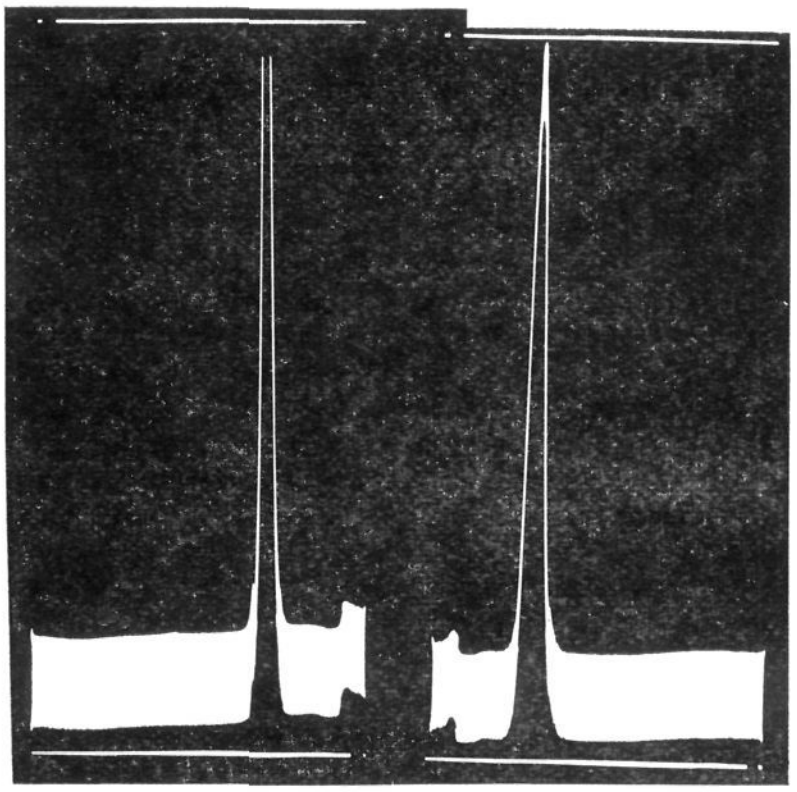

Fig. 1.—Electrophoretic diagram of taka amylase: acetate buffer, *p*H 6.0, $\mu = 0.2$, 0°, 2940 seconds at 128 v./sec., mobility -4.9×10^{-5} cm.^{2}/sec./volt. Ascending boundary is to the right.

Stability.—Taka amylase is a relatively stable enzyme. Aqueous solutions of highly purified or of crystalline taka amylase retain their amylase activity upon standing for extended periods of time provided the *p*H and temperature are favorable. For example, 1 to 5% aqueous solutions of preparations of highly purified maltase-free taka amylase and also of crystalline taka amylase showed no loss of amylase activity upon standing at *p*H values of 5.8 to 7.2 and at 5 to 10° for more than a year. On the other hand, such solutions lose their amylase activity rapidly when they are adjusted to unfavorable hydrogen ion activities, or held at unfavorable temperatures. Thus, for any given temperature and concentration, solutions of taka amylase are less stable at *p*H 2.9 to 3.4 than at *p*H 5.8 to 7.2. Similarly, for any given *p*H value or amylase concentration, solutions of taka amylase are less stable the higher the temperature. These points are illustrated by the data given in Table I. Fischer and de Montmollin[10] report that taka amylase is unstable below *p*H 5.5 or above *p*H 8.5. Under otherwise favorable conditions, taka amylase retains its amylase activity upon extensive dilution with water and upon standing in dilute aqueous solution (7.6×10^{-4} mg. per ml.). However, under otherwise unfavorable conditions the inactivation of taka amylase increases with dilution.

Influence of Calcium Ions upon Stability of Taka Amylase.—The data given in Table I illustrate

(10) E. H. Fischer and R. de Montmollin, *Helv. Chim. Acta*, **34**, 1994 (1951).

(11) These analyses were carried out by Dr. Adalbert Elek at the Elek Microanalytical Laboratories, Los Angeles, California.

(12) The emission spectrographic analyses were carried out by Mr. J. Dunbar in the Laboratory of Professor T. I. Taylor.

the fact that taka amylase, like several other alpha amylases,[6,13-16] is protected, to a greater or to a smaller extent, from inactivation under unfavorable conditions by the presence of calcium ions. Thus, an aqueous solution of taka amylase containing 0.053 mg. per ml. lost all of its activity in 120 minutes at 60° at *p*H 7.0 but only 21% of its activity under the same conditions in the presence of 0.02 *M* calcium chloride. The loss of taka amylase activity observed at *p*H 2.9 to 3.4 at 0 to 10° also is decreased but not prevented entirely by the presence of calcium ions. It is interesting to note that the unfavorable influence of high hydrogen ion activities of its solutions upon the stability of taka amylase resembles that observed for malt alpha amylase by Ohlsson.[17] Moreover, the protective action of calcium ions on taka amylase is similar to that observed for malt alpha amylase by Kneen[13] but is in contrast to the unfavorable influence of calcium ions on the stability of malt beta amylase.[14] Rau and Sreenivasan[18] also have stated that calcium ions protect taka amylase from inactivation.

The data given in Table II show that the protection of taka amylase from inactivation under unfavorable conditions observed in the presence of added calcium salts is due to a specific influence of the calcium ions and that equivalent concentrations of sodium ions, acetate ions or chloride ions cannot replace the calcium ions. Sodium ions, acetate ions and chloride ions exert no protective action on the stability of taka amylase under the conditions studied. An explanation for the protective action of calcium ions on the stability of taka amylase is being sought.

TABLE I

FAVORABLE INFLUENCE OF CALCIUM IONS UPON THE STABILITY OF TAKA AMYLASE IN AQUEOUS SOLUTION

Treatment of amylase[a] solution					
Concn., mg./ml.	*p*H	Calcium ions, mole/l.	Temp., °C.	Time, min.	Amylase activity remaining,[b] %
0.244	3.1	0	0–10	2880	16
.244	2.9	0.02	0–10	2880	27
.244	3.4	0	5–10	2880	50
.244	3.4	0.02	5–10	2880	78
.244	3.4	0	50	15	0
.244	3.4	0.02	50	15	0
.244	5.8	0	5–10	2880	95
.244	5.8	0.02	5–10	2880	100
.244	5.8	0	50	15	95
.244	5.8	0.02	50	15	100
.244	5.8	0	50	30	70
.244	5.8	0.02	50	30	96
.244	5.8	.02	50	90	92
.244	5.8	.02	60	30	84
.244	5.8	.02	60	60	76
.244	5.8	.02	60	90	70
.244	5.0	.02	60	8	90
.244	5.0	.02	60	30	19
.244	7.0	.02	60	30	82
.244	7.0	.02	60	60	75
7.6×10^{-4}	5.0	0	40	30	65
7.6×10^{-4}	5.0	0.02	40	30	94
3.05×10^{-3}	6.5	0	60	60	0
3.05×10^{-3}	6.5	0.02	60	60	72
0.053	7.0	0	60	120	0
0.053	7.0	0.02	60	120	79

[a] Highly purified maltase-free taka amylase. [b] After treatment, portions of each amylase solution were measured for amylase activity[6] with Lintner's soluble potato starch: 1%; 0.01 *M* acetate; 0.02 *M* calcium chloride; *p*H 5.0; 40°.

TABLE II

A COMPARISON OF THE INFLUENCE OF CALCIUM IONS AND OF CERTAIN OTHER IONS ON STABILITY OF TAKA AMYLASE IN AQUEOUS SOLUTIONS

Amylase solution treatment[a]				
Buffer: sodium acetate, acetic acid, moles/l.	Additional salt Kind	*M*	Temp., °C.	Amylase activity remaining,[b] %
0	0	0	0	98
0	0	0	60	0
0.02	0	0	0	99
.02	0	0	60	0
.02	Calcium acetate	0.02	0	98
.02	Calcium acetate	.02	60	79
.02	Sodium chloride	.04	0	100
.02	Sodium chloride	.04	60	0
.02	Calcium chloride	.02	0	100
.02	Calcium chloride	.02	60	78

[a] Solution of recrystallized maltase-free taka amylase; 6.1×10^{-3} mg. amylase per ml.; *p*H 5.8; held for 60 minutes, at 0 or 60°, as indicated. [b] After treatment, portions of each amylase solution were measured for amylase activity[6] with Lintner's soluble potato starch; 1%, 0.01 *M* acetate, 0.02 *M* calcium chloride; *p*H 5.0; 40°.

Failure of Calcium Ions to Activate Taka Amylase.—The data given in Table III lead to the conclusion that calcium ions do not activate taka amylase but that any favorable influence they exert is due to protection of the amylase from inactivation under unfavorable conditions. Thus, a freshly prepared

TABLE III

FAILURE OF CALCIUM IONS TO ACTIVATE TAKA AMYLASE

Amylase solution treatment[a]		Conditions of hydrolysis[b]	
Calcium acetate, moles/l.	Temp., °C.	Calcium acetate, moles/l.	Amylase activity remaining, %
0	No standing	0.02	100
0	No standing	0	97
0	0	0.02	99
0	0	0	94
0.02	40	0.02	94
0	40	0.02	66
0	40	0	61

[a] Solution of recrystallized maltase-free taka amylase: 1.22×10^{-3} mg. amylase per ml.; *p*H 5.0; held for 30 minutes at 0° or at 40° in presence or in absence of calcium acetate, as indicated. [b] After treatment, portions of each amylase solution were measured for amylase activity with Lintner's soluble potato starch, 1%; 0.01 *M* acetate; *p*H 5.0; 30 minutes; 40° in the presence or in the absence of calcium ions, as indicated; 6.1×10^{-2} mg. amylase per gram substrate,

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(14) S. Schwimmer and A. K. Balls, *J. Biol. Chem.*, **176**, 465 (1948).

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TABLE IV

EVIDENCE THAT TAKA AMYLASE CAN BE FREED FROM TRACES OF EXTRANEOUS MALTASE

Relative concn. of taka amylase, units[a]	Hydrolysis of maltose[b] Time in hours 0.5	2	24
	Theoretical glucose, %		
A. Before selective inactivation of maltase in amylase solution[c]			
1	0	0	0
10	0	0	0
50	0	0	13.4
100	0	5.5	31.4
500	3.8	8.1	90.0
B. After selective inactivation of maltase in amylase solution[c]			
100	0	0	0
C. Crystalline taka amylase			
100	0	0	0

[a] Unit concentration; 0.089 mg. taka amylase preparation per gram substrate, required to cause 20% hydrolysis in 30 minutes at 40° of 1% Lintner's soluble potato starch: 0.01 *M* acetate; 0.05 *M* sodium chloride; *p*H 5.0; 40°.[4,6] [b] Maltose: 1%; 0.01 *M* acetate; 0.05 *M* sodium chloride; *p*H 5.0; 40°.[4,6] [c] Treatment of amylase solution: 10 mg. purified amylase preparation per ml.; 0.02 *M* calcium chloride; *p*H 5.8; held at 60° for 20 minutes: cooled rapidly to 0°.

solution of crystalline taka amylase showed no significant difference in its amylase activity when it reacted with starch at 40° in the presence or in the absence of added calcium ions. Similarly, the presence or the absence of calcium ions in the substrate had very little influence on the amylase activity of a solution of taka amylase that had lost approximately 30% of its amylase activity by being held for 30 minutes at 40°. Incidentally, the data show that this loss of amylase activity was largely prevented by the presence of calcium ions in the amylase solution while it was being held for 30 minutes at 40° and before it was measured for amylase activity. The comparisons of the amylase activities of the amylase solutions held for 30 minutes at 40° and at 0° indicate that some loss of amylase activity occurs during the 30 minute hydrolysis of the starch at 40° but that this loss is prevented largely by the presence of the substrate. A similar protection of pancreatic amylase by its substrate has been reported.[8] Fischer and de Montmollin[10] also state that calcium ions do not activate taka amylase. Pancreatic amylase, like taka amylase, is protected from inactivation[8,15] but is not activated by calcium ions.[15]

Removal of Traces of Maltase Impurities.—Taka amylase is accompanied in extracts of *Aspergillus oryzae* by a number of other enzymes. These include maltase. The data given in Table IVA show that highly purified preparations of taka amylase obtained as described previously[3,4] retained slight traces of maltase activity that might become significant in the interpretation of the results if sufficiently high concentrations of the amylase preparations were used for sufficiently long periods of time to study the products formed in the

TABLE V

SELECTIVE INACTIVATION OF MALTASE IN SOLUTIONS OF HIGHLY PURIFIED TAKA AMYLASE

Treatment of amylase solution					Activities of amylase solution after treatment				
Concn. of amylase preparation, mg./ml.	*p*H	Calcium chloride, moles/l.	Temp., °C.	Time, min.	Saccharogenic activity[a] (S) recovered, %	Amyloclastic activity[b] (A) recovered, %	Ratio *A/S*	Maltase activity[c] (M) recovered, %	Ratio *S/M*
A. Influence of unfavorable hydrogen ion activity									
12.2	7.2	None	5–10	2880	100	100	6.2	100	74
0.244	3.4	None	5–10	1440	69	73	6.5	82	63
.244	2.9	0.02	5–10	2880	27	36	8.0	100	20
.244	3.1	None	5–10	4320	3			93	2.4
.244	2.9	0.02	5–10	4320	1			82	0.8
B. Selective inactivation at 50°									
0.244	5.8	0.02	50	0	100	100	6.7	100	84
.244	5.8	.02	50	15	101	100	6.6	69	122
.244	5.8	.02	50	45	94	94	6.7	65	119
.244	5.8	.02	50	60	93	94	6.8	69	113
.244	5.8	.02	50	90	92	94	6.9	69	111
C. Selective inactivation at 60° in relatively dilute solution									
0.244	5.8	0.02	0	60	100	100	6.4	100	65
.244	5.8	.02	60	5	100	100	6.4	58	113
.244	5.8	.02	60	15	97	100	6.6	24	260
.244	5.8	.02	60	30	84	88	6.7	21	258
.244	5.8	.02	60	60	76	83	6.9	6	815
D. Selective inactivation at 60° in highly concentrated solutions									
9.76	5.8	0.02	60	20	107	118	7.0	0	∞
11.2	5.8	.02	60	20	97	108	7.0	0	∞

[a] Mg. maltose equivalents per mg. amylase preparation in 30 minutes at 40° from Lintner's soluble potato starch: 1%; 0.01 *M* acetate; 0.05 *M* sodium chloride; *p*H 5.0.[6] [b] Mg. starch hydrolyzed per mg. amylase preparation in 30 minutes at 40° to products that give a clear red color with iodine in KI from Lintner's soluble potato starch: 1%; 0.01 *M* acetate; 0.05 *M* sodium chloride; *p*H 5.0.[6] [c] Mg. glucose produced per mg. amylase preparation in 24 hours at 40° from 1% maltose: 0.01 *M* acetate; 0.05 *M* sodium chloride; *p*H 5.0.

TABLE VI

EVIDENCE THAT AMYLASE AND DEXTRINASE ACTIVITIES BOTH ARE PROPERTIES OF THE SAME ENZYME, TAKA AMYLASE

Treatment of amylase[a] solution					Activities of amylase solution after treatment				
					Saccharogenic activity[b]		Dextrinase activity[c]		
Concn. of amylase preparation, mg./ml.	*p*H	Calcium chloride, moles/l.	Temp., °C.	Time, min.	Units	% of original	Units	% of original	Ratio *S/D*
12.2	7.2	None	0	0	2200	100	220	100	10.0
0.244	5.8	0.02	50	15	2020	92	203	92	9.9
.244	5.8	None	50	15	1860	85	188	85	9.9
.244	3.4	None	5–10	1440	1400	64	144	65	9.7
.244	2.9	None	5–10	2880	566	26	50	23	11.3

[a] Highly purified maltase-free preparation of taka amylase. [b] Mg. maltose equivalents in 30 minutes at 40° from Lintner's soluble potato starch: 1%; 0.01 *M* acetate: 0.05 *M* sodium chloride; *p*H 5.0. [c] Mg. maltose equivalents in 20 hours at 40° from dextrins: 1%; 0.01 *M* acetate; 0.05 *M* sodium chloride; *p*H 5.0. Dextrins were products remaining when hydrolysis of Lintner's soluble potato starch had reached 50% theoretical "maltose" by action of taka amylase under the conditions of hydrolysis given in [b], above.

hydrolysis of starches or of their components. The data given in Table IVB and IVC show that these traces of maltase impurity can be removed by selective inactivation or by crystallization leaving active maltase-free taka amylase. Fischer and de Montmollin also report that crystalline taka amylase has no maltase activity.[5]

Differences in Stabilities of Taka Amylase and of Taka Maltase.—The data summarized in Table V compare the stabilities of taka amylase and of taka maltase under different conditions and give additional evidence that the highly purified preparations of taka amylase[3,4] contain only one amylase contaminated by traces of extraneous maltase. Thus, while the ratio of two manifestations of amylase action, that of the amyloclastic activity[6] to the saccharogenic activity,[6] remains substantially constant under the different conditions studied, the ratios of amylase activity (starch as substrate) to maltase activity (maltose as substrate) change markedly, showing selective inactivation of maltase or of amylase.

It is evident from the data given in Table V that calcium ions do not protect taka maltase as efficiently as they do taka amylase from inactivation at higher temperatures. Therefore, it was possible, as shown in Table IV, to free highly purified taka amylase from the last traces of maltase impurities by selective inactivation at higher temperatures in much the same way that Ohlsson[17] used to free malt alpha amylase from contamination with malt beta amylase but aided in this case by the presence of calcium ions to protect the amylase. In order further to minimize the loss of taka amylase during the inactivation of taka maltase, it was found advisable, as indicated in Table V, D, to use relatively concentrated solutions of taka amylase preparations. Therefore, the solutions used for the inactivation of the maltase contained 10 mg. amylase preparation per ml. These solutions were adjusted to 0.02 *M* calcium chloride, 0.01 *M* acetate and *p*H 5.8, held at 60° for 20 minutes and then chilled rapidly. As shown in Table IVB solutions of taka amylase that had been treated in this way showed no trace of maltase activity even when 100 times the unit concentration of the amylase reacted with 1% maltose at 40° for 24 hours.

Dextrinase Activity, a Property of Taka Amylase. In contrast to the evidence for a maltase impurity that could be removed (Tables IV and V), the highly purified maltase-free preparations of taka amylase failed to show any evidence of the presence of extraneous dextrinase activity. Thus, the data given in Table VI show that the ratio of amylase activity (starch as substrate) to dextrinase activity (dextrins as substrate) remains constant under a number of conditions that cause marked losses of amylase and of dextrinase activities and that might be expected to cause the selective inactivation of the amylase or of the dextrinase if these activities were due to separate enzymes. Similarly, the data given in Table VI also confirm and extend the evidence given in Table V that the purified preparations of taka amylase contain only one amylase.

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