CCLIV. THE PRESENCE OF A KINASE OF AMYLASE IN TRYPSIN PREPARATIONS.

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FORD and GUTHRIE [1908], examining extensively the influence of papain on the amount of amylase in barley, make the general statement that of all proteolytic enzymes papain acts the best. Wróblewski [1902], Biedermann [1922] Fricke and Kaja [1924] declare that trypsin has no influence on malt amylase. We, therefore, thought it necessary to examine more thoroughly the influence of trypsin on the amount of amylase in barley, since preliminary tests had proved its specific action.

Several kinds of barley were taken for the experiments. 25 g. of ground barley were immersed in 150 ml. H_2O , to which 4 ml. of toluene were added. The extracts were made in two different ways: one was mechanically shaken during 14 hours at 20° , the other was only occasionally stirred at the same temperature and for the same length of time. The action of trypsin was examined by the addition of 0.5 g. or 0.8 g. to 25 g. of barley. Extracts were also made with the addition of 3 g. of papain, which amount, as our researches have proved, produces a maximum of amylase in barley [1934]. After filtration of the extracts, the three amylolytic functions of the amylase were determined.

The saccharifying power was investigated after dilution in the proportion 10 : 100, and in extracts prepared with an addition of proteolytic enzymes after a dilution of 5 : 100. 5 ml. of the diluted extract were added to 50 ml. of a 2.5 % solution of Merck's soluble starch, the p_H was adjusted to 4.87 and the mixture kept at 50° for 15 minutes. Then 10 ml. of $N/10$ NaOH were added. After cooling, reducing sugars were determined by the method of Willstätter and Schudel [1918]. The results obtained shown in the tables correspond to the amount of reducing sugar, expressed in ml. $N/20$ iodine, which was produced under the above conditions by a solution corresponding to 1 g. of barley.

The dextrinising power was investigated by adding to 50 ml. of the above starch solution 5 ml. of undiluted extract at 50° ; every 4 mins. 1 ml. of the mixture was taken out, added to 1 ml. of iodine solution (0.8 g. iodine in 1 litre H_2O) and the colour observed. The dextrinising activity was measured by the time required for obtaining the following colours: violet, brownish red, light yellow.

The liquefying power was established by the method of Pollak-Chrząszcz-Pierożek [Chrząszcz and Pierożek, 1910]. It is expressed in the tables by the amount of starch which 1 g. of barley can liquefy under the above conditions.

The proteolytic power of trypsin preparations (Merck) was established by the Waldschmidt-Leitz method [1924]. A solution was taken in which 10 mg. of trypsin were present in 1 ml. of the solution, which acted on a mixture of 5 ml. 3 % solution of gelatin + 1 ml. buffer p_H 8.9 at 30° for 60 mins., and the proteolytic activity was expressed in ml. of 0.2 N KOH.

The influence of trypsin on the amount of amylase in barley.

To investigate this the following extracts were made:

No. 1 and 4. 25 g. of barley I + 150 g. H₂O + 4 ml. toluene.

No. 2 and 5. As No. 1 + 0.5 g. trypsin.

No. 3 and 6. As No. 1 + 0.8 g. trypsin.

Extracts Nos. 1–3 were shaken occasionally during 14 hours.

Extracts Nos. 4–6 were mechanically shaken for 14 hours.

No. 7. 0.5 g. of trypsin was dissolved in 150 ml. of H₂O directly before testing.

No. 8. 0.8 g. of trypsin was dissolved in 150 ml. of H₂O directly before testing.

Table I.

No. of solution	Saccharifying power. Sugar produced by 1 g. barley (ml. N/20 I)	Increase %	Dextrinising power			Liquefying power. 1 g. of barley liquefies starch (g.)	Increase %
			Violet	Brownish red	Light yellow		
				was obtained with iodine in mins.			
1	356	—	105	Not obtained in 1200 mins.		3.0	—
2	488	37	5	10	30	9.0	200
3	498	40	2	5	20	9.0	200
4	236	—	120	Not obtained in 1200 mins.		2.6	—
5	468	98	5	12	35	9.0	246
6	498	110	2	5	20	9.0	246
7	4.5	—	60	105	Not obtained in 1200 mins.	0.3	—
8	8.1	—	20	40	600	0.4	—

These experiments prove that trypsin increases the amylolytic activity of barley, though it does not affect every function to the same degree. The dextrinising power is most strongly affected. With a barley water extract prepared without any additions the violet colour is reached only after 100 mins. and this stage even after 20 hours is not much changed; whilst an extract prepared with addition of trypsin reaches the first stage of decomposition of the starch in 2 to 5 mins., and the decomposition to the achro-stage in 20 or 30 mins. is particularly accelerated. Though trypsin by itself has a dextrinising effect, the first and second stages of the decomposition of the starch are 10 times slower, and the achro-stage is not reached even after 20 hours when 0.5 g. trypsin is used. The liquefying power, though being considerably increased, is still weak. The saccharifying power is likewise greatly increased but less in proportion to the dextrinising power. An addition of 0.8 g. of trypsin to 25 g. of barley produces a maximum of saccharifying activity.

Attention should be drawn to the fact that extracts obtained by long constant shaking give worse amylolytic results than extracts prepared by shaking only occasionally. A small addition of trypsin diminishes the difference resulting from the mode of preparation of the extract, whilst a larger quantity of trypsin reduces this difference almost to nil, thus proving that trypsin protects amylase against inactivation caused by shaking and thus acts simultaneously as an eleutosubstance [Chrzaszcz and Janicki, 1933].

The influence of trypsin free from amylase on the quantity of amylase in barley.

The trypsin preparation as seen in Table I contains a small quantity of amylase, and as it was interesting to investigate how a preparation free from amylase would act on barley, we undertook to free trypsin from it. After

several preliminary tests, it became evident that the easiest way of removing amylase is by autodigestion of the trypsin preparation at 40°. A solution was prepared of 0.8 g. of trypsin in 150 ml. of water and 4 ml. of toluene and was heated for 1 hour at 40°. Of this heated trypsin solution 5 ml. were added to the starch solution but showed no signs of either saccharifying or dextrinising power. The colour of the starch solution with iodine was blue after 20 hours of action, and the liquefying power was minimum. Though a greater concentration of trypsin or the use of larger quantities of the solution still showed some traces of dextrinising power, we did not make use of such amounts in our researches. Since the quantities of the trypsin used by us do not show any definite signs of amylase, we consider the solution treated as above as free from amylase and shall refer to such a solution as "trypsin free from amylase." The proteolytic power of the digested trypsin solution compared with the undigested was as follows:

10 mg. of trypsin native	...	...	...	0.9 ml. 0.2N KOH
10 mg. of trypsin free from amylase	...	...	...	0.7 ml. 0.2N KOH.

In order to ascertain the activating power of trypsin free from amylase on the amylase in barley and to compare this with that of papain or peptone the following extracts were made up:

No. 1 and 5. 25 g. of barley II + 150 g. H₂O + 4 ml. toluene.

No. 2 and 6. As No. 1 + 3 g. papain.

No. 3 and 7. As No. 1 + 0.8 g. trypsin native.

No. 4 and 8. As No. 1 + 0.8 g. trypsin free from amylase.

No. 9. As No. 1 + 3 g. papain inactivated by boiling.

No. 10. As No. 1 + 0.8 g. trypsin inactivated by boiling.

No. 11. As No. 1 + 3 g. peptone.

Extracts Nos. 1-4 were shaken only occasionally during 14 hours.

Extracts Nos. 5-11 were obtained by continuous mechanical shaking.

Table II.

No. of solution	Saccharifying power. Sugar produced by 1 g. barley (ml. N/20 I)	Increase %	Dextrinising power			Liquefying power. 1 g. of barley liquefies starch (g.)	Increase %
			Violet	Brownish red	Light yellow		
was obtained with iodine in mins.							
1	233	—	105	Not obtained in 1200 mins.		3	—
2	498	114	80	"		9	200
3	468	101	2	10	" 20	9	200
4	450	93	12	89	350	8	167
5	117	—	120	Not obtained in 1200 mins.		2.6	—
6	433	270	92	"		9	246
7	394	237	2	10	" 20	9	246
8	380	225	15	102	370	8	208
9	128	10	110	Not obtained in 1200 mins.		7	170
10	125	7	110	"		7	170
11	153	30	100	"		8	208

The above investigations show that trypsin free from amylase increases the amylolytic power of the extract of barley, but in a less degree than native trypsin, and that the dextrinising power is liable to greater loss of activity than the saccharifying power. Papain acts a little more efficaciously than trypsin on the saccharifying power, whilst the dextrinising power is only very slightly affected. Trypsin and papain inactivated by boiling act only as eleutosubstances, though in a less degree than peptone. It should be noted still that the barley

in Table II shows only a rather moderate increase of amylolytic power under the influence of trypsin, whilst some barleys can be found whose increase of this power, and particularly of the dextrinising function, is considerably greater. Thus with an extract of barley prepared by addition of trypsin free from amylase we obtained the achro-stage in 180 mins.

Continuous shaking of the extracts has proved to be less advantageous than preparation of the extracts by stirring periodically, even when trypsin free from amylase or papain has been added. On the other hand, if we add to the extract of barley, prepared by shaking continuously, some peptone or inactivated solutions of enzymes, they play then the rôle of an eleutosubstance and thus partly protect the amylase against the negative influence of shaking. The influence of papain and peptone in this respect has been discussed elsewhere by us [1934].

The presence of a kinase of amylase in trypsin preparations.

The phenomenon of a very marked increase of the dextrinising activity of extracts of barley prepared by addition of trypsin has given rise to the following question: Is the action of trypsin similar to that of papain, *i.e.* probably the liberation of the amylase by the enzymic decomposition of protein, which binds it and keeps it therefore in an inactive form, or is it another kind of activation?

In order to investigate these questions the following extracts and solutions were made.

No. 1. 25 g. of barley II + 150 g. H_2O + 4 ml. toluene, mechanically shaken during 14 hours.

No. 2. As No. 1 + 3 g. of papain.

No. 3. Filtered extract No. 1 + solution of inactivated trypsin (0.8 g. + 150 ml. H_2O boiled) in the proportion 1 : 1.

No. 4. Filtered extract No. 1 + solution of trypsin free from amylase in the proportion 1 : 1.

No. 5. Filtered extract No. 2 + solution of inactivated trypsin as in No. 3.

No. 6. Filtered extract No. 2 + solution of trypsin as in No. 4.

To ascertain the dextrinising power of solutions Nos. 3–6 10 ml. were used which contained 5 ml. of primary extract of barley. The saccharifying power was determined as usual in suitably diluted solutions.

Table III.

No. of solution	Saccharifying power. Sugar produced by 1g. barley (ml. N/20 I)	Increase %	Dextrinising power		
			Violet	Brownish red	Light yellow
			was obtained with iodine in mins.		
1	233	—	120	Not obtained in 1200 mins.	
2	498	—	105	"	"
3	233	0.0	105	"	"
4	233	0.0	10	60	300
5	498	0.0	85	Not obtained in 1200 mins.	
6	498	0.0	8	50	240

From Table III it is evident that the trypsin solution influences only the dextrinising function of the amylase in solution, and this in a very high degree, as the filtrate of the aqueous extract of barley, or the extract made up by addition of papain to a solution of trypsin free from amylase, shows the same, or even a slightly greater, dextrinising power as the extract of barley prepared with the addition of trypsin free from amylase. (Compare the dextrinising

powers of extracts No. 4 and 8 Table II. with the dextrinising powers of solutions 4 and 6 of Table III.) The saccharifying power of the solutions with trypsin free from amylase remains, however, unchanged. We can conclude, therefore, that the increase of the dextrinising function under the influence of trypsin is independent of the increase of the saccharifying function of the amylase of barley under the influence of the same trypsin, and that the increase of these two functions must result from two different agents. The increase of the saccharifying power of the extract of barley with addition of trypsin free from amylase appears in conditions analogous to those in which this function increases under the influence of papain, therefore the factor effecting this increase must be the same in both cases. This factor is the proteolytic action of trypsin, causing either the decomposition of protein binding this functional group of the amylase, or the formation of new quantities of amylase. This was explained in our preceding papers concerning the action of papain on the increase of the amount of amylase in corn [1934]. The dextrinising function, however, proves to be quite different. A solution of papain does not, as we have shown, affect a solution of amylase, whilst a trypsin solution produces under the same circumstances a large activation of the dextrinising power. It follows that the increase of the dextrinising function of the extract of barley prepared with an addition of trypsin free from amylase does not appear under the influence of the proteolytic action, but of a special agent found only in a preparation of trypsin and not in papain. This particular agent causing an increase of the dextrinising function of the amylase of barley is a special dextrinising kinase, present in the trypsin preparations and its solutions.

In the above-mentioned researches we have shown that our kinase acts on the dextrinising power. It was therefore interesting to investigate whether a considerably increased dose of trypsin containing kinase would not also stimulate the saccharifying power. Experiments were made with aqueous extracts of barley and an extract prepared with an addition of papain to which was added after filtration a solution of trypsin free from amylase in the proportion 1 : 19; an increase of the saccharifying power resulted, amounting in the first case to 66 %, and in the second, prepared with addition of papain, only to 25 %. Since the presence of amylase is evident when using considerable quantities of native trypsin, and the stimulation of the saccharifying function under the influence of large quantities of kinase can be considered in such conditions as certain, yet the problem of the power of the kinase relative to this function should be set aside until pure kinase has been obtained.

If we look from the standpoint of our observations at the amylase problem as represented now in the literature, above all the existence of a special dextrinogen amylase, as understood by Ohlsson, seems questionable. Furthermore the assertion of the same author, that dextrinogen amylase is produced only during the germination of the corn, is also very doubtful. For the present, the most one can suppose is that kinase arises during the germination of the corn, and according to the quantity of this kinase the dextrinising function of the amylase increases. On the basis of the results obtained it is difficult to understand how the kinase acts on amylase and in what relation it stands to the amylokinase of Waldschmidt-Leitz [1931], found by him in germinated barley, and to Weidenhagen's [1933] point of view on this amylokinase. According to Waldschmidt-Leitz his amylokinase should above all act on the saccharifying power. The researches of Reichel [1932] show, however, that this amylokinase is also able to stimulate the dextrinising function of amylase. We hope further investigations will solve these problems.

SUMMARY.

1. Trypsin produces a considerable increase in the amylolytic power of barley, both in the saccharifying and in the dextrinising functions, the liquefying function remaining minimum.

2. The influence of trypsin on the increase of the amylolytic power of barley differs from the influence of papain mainly as regards the dextrinising function. This function under the action of papain is only slightly increased, under the influence of trypsin very markedly. The saccharifying function is slightly more increased under the influence of papain than under that of trypsin.

3. The cause of the increase of the quantity of amylase in barley under the influence of trypsin is twofold.

(a) Similar to the action of papain on barley, *i.e.* causing either the decomposition of protein combined with amylase, or the formation of new quantities of amylase. Under the influence of this action we have above all an increase of the saccharifying function.

(b) With trypsin we have another action, namely the influence of a special kinase of amylase found in trypsin preparations. Under the action of this kinase the dextrinising power is above all strongly increased and the saccharifying power only a little.

4. Both in the active and inactivated trypsin, eleutosubstances are also found. These substances protect the amylase partly against inactivation by shaking, just as does peptone or inactivated papain, though in a less degree.

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