CLXVI. THE PROTEOLYTIC ENZYMES OF YEAST.

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An investigation into the action of proteolytic enzymes on the oxytocic principle of the pituitary gland [Gulland and Macrae, 1933] required the preparation of the yeast proteolytic enzymes—dipeptidase, aminopolypeptidase and proteinase. In recent years Willstätter, Grassmann and their colleagues have described excellent methods for the preparation of these enzymes from Löwenbräu yeast, whereby complete separation of the enzymes has been achieved [1926; 1927; 1928, 1, 2, 3; 1930]. Since the strain of yeast used by these authors could not be obtained in a fresh condition, it was hoped to devise methods for the preparation of those enzymes from the more readily available English brewer's top-yeast.

It is well known that yeasts from different sources may not behave identically, and it was therefore to be expected that English and German yeasts might liberate their enzymes at different rates. Since the methods used by the German authors depended largely on the fractional autolysis of the dead yeast cells, it was improbable that their methods could be applied without modification to the preparation of the enzymes from English yeast. A brewer's top yeast was used for the greater part of the work now described, but a few experiments were carried out with a Dutch baker's yeast; Löwenbräu yeast, on the other hand, is a brewer's bottom yeast. A complete investigation of the problem has not been attempted, but the results obtained may be of value to others who may require these important enzymes.

Qualitatively the English and Dutch yeasts behaved like Löwenbräu yeast, but marked quantitative differences were observed. By modifying the method of the German authors, dipeptidase preparations were obtained containing only traces of aminopolypeptidase and completely free from proteinase. Aminopolypeptidase was prepared by a slight modification of the existing method and was completely free from dipeptidase and proteinase. The purification of proteinase, however, has offered much greater difficulties. Using English top-yeast and Dutch baker's yeast, no method gave a proteinase preparation which was free from aminopolypeptidase, although the proportion of proteinase to aminopolypeptidase was always much higher than that of normal yeast autolysates. Proteinase preparations have been obtained completely free from dipeptidase.

Experimental.

Methods of assaying enzyme preparations.

The procedure used for the assay of dipeptidase, aminopolypeptidase and proteinase was the method of alkali titration in 90 % alcohol [Willstätter and Waldschmidt-Leitz, 1921]. The activities of dipeptidase and aminopolypeptidase

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are estimated in units which are defined below. The activity of proteinase is estimated as the increase in acidity of a gelatin solution in unit time (see below).

The activity of dipeptidase is estimated using *dl*-leucylglycine as substrate, the unit [Willstätter and Grassmann, 1926] being that quantity of enzyme which hydrolyses one half of the *l*-peptide in 1 hour at p_H 7·8 and 40° when acting on 225 mg. of *dl*-leucylglycine in 10 cc. of *M*/30 phosphate buffer. The estimation of dipeptidase was carried out in the following manner. A solution prepared in a graduated flask (2·5 cc.) by warming 56 mg. of *dl*-leucylglycine with *M*/3 phosphate buffer at p_H 7·8 (0·25 cc.) and water (about 1 cc.) was cooled and brought to p_H 7·8 with about 2 drops of *N* ammonia. The enzyme solution was then added, and the contents of the flask diluted to the mark with water and mixed. 1·0 cc. of the mixture was immediately transferred to a flask containing absolute alcohol (9 cc.) and 4 drops of a 0·5 % alcoholic solution of thymolphthalein and titrated with *N*/20 KOH in 90 % alcohol until a faint blue colour was just perceptible. The graduated flask containing the remainder of the solution had meantime been placed in a thermostat at 40° and after 1 hour, 1·0 cc. of the solution was removed and titrated in the same way. The difference between the titration values represented the degree of hydrolysis. An increase in acidity equivalent to 0·60 cc. of *N*/20 KOH corresponds with 50 % hydrolysis of the *l*-peptide and is effected by 0·25 unit of dipeptidase. The amount of enzyme taken must be adjusted so that not less than 25 % and not more than 75 % of the *l*-peptide is hydrolysed.

The activity of aminopolypeptidase is estimated using *dl*-leucylglycylglycine as substrate, the unit [Grassmann and Dyckerhoff, 1928, 3] being that quantity of enzyme which hydrolyses one half of the *l*-peptide in 1 hour at p_H 7·0 and 40°, when acting on 245 mg. of *dl*-leucylglycylglycine in *M*/30 phosphate and *M*/25 ammonium chloride having a total volume of 10 cc. The determination of aminopolypeptidase was carried out in a similar manner to that of dipeptidase. A solution of 61 mg. of *dl*-leucylglycylglycine in *M*/3 phosphate at p_H 7·0 (0·25 cc.) and *M*/2·5 ammonium chloride-ammonia at p_H 7·0 (0·25 cc.) was adjusted to p_H 7·0 with a trace of *N* ammonia; no warming on the water-bath was required, since leucylglycylglycine is easily soluble in water. The enzyme was added and, after the solution had been diluted to 2·5 cc., 1·0 cc. portions were removed at intervals of 1 hour (temperature 40°) and titrated in the same manner as in the determination of dipeptidase. An increase in acidity equivalent to 0·50 cc. of *N*/20 potassium hydroxide corresponds with 50 % hydrolysis of the *l*-peptide and is effected by 0·25 units of aminopolypeptidase. The amount of enzyme used must effect hydrolysis of not less than 25 % and not more than 75 % of the *l*-peptide. In the presence of dipeptidase the results of the determination are too high since there is some hydrolysis of the glycylglycine formed by the action of the aminopolypeptidase.

The estimation of proteinase was carried out as follows. A mixture of a 10 % gelatin solution (6 cc.) and *M*/5 citrate buffer at p_H 5·0 (1·6 cc.) in a 10 cc. graduated flask was adjusted to p_H 5·0 with a trace of *N* acetic acid. The enzyme solution was then added, and the contents of the flask were diluted to the mark with water and immediately mixed thoroughly by being warmed in a thermostat at 40° and shaken gently. A 2 cc. portion was at once removed and added to absolute alcohol (18 cc.) which had previously been heated to 50–60°. Precipitation of gelatin occurred, and after the addition of 6 drops of thymolphthalein the mixture was titrated with *N*/20 KOH in 90 % alcohol until a permanent faint blue colour had developed; constant shaking was essential during the titration. After the hydrolysis had proceeded for 24 hours at 40°, a

further 2 cc. portion was removed and titrated. The results are quoted as increases in acidity. In presence of aminopolypeptidase high values are obtained owing to the action of that enzyme on the products of the proteinase hydrolysis of gelatin.

Dipeptidase.

Grassmann and Klenk [1930] obtained "pure" preparations of dipeptidase from Löwenbräu yeast by autolysis of the dead yeast cells for 20 hours at p_H 6·4–6·8. The autolysate thus obtained was very rich in dipeptidase and also contained some aminopolypeptidase and proteinase. Rapid adsorptions at p_H 5 with small quantities of specially prepared suspensions of aluminium hydroxide and kaolin removed all the proteinase and some of the aminopolypeptidase, but left most of the dipeptidase, which was finally obtained free from aminopolypeptidase by careful fractional precipitation with acetone. The direct application of this method to the English top-yeast invariably yielded preparations which had much dipeptidase activity, were free from proteinase, but contained some aminopolypeptidase; in the many experiments which were carried out, two different preparations of aluminium hydroxide Cγ gave similar results. The weights of the dry preparations obtained were only about one half of those recorded by Grassmann and Klenk. The enzyme activities of the preparations varied to some extent, but the following were typical preparations; proteinase activity was entirely absent.

Table I.

	Units of dipeptidase per 10 mg.	Units of aminopolypeptidase per 10 mg.
1	0·48	0·20
2	0·51	0·18
3	0·62	0·23

An attempt to free one of the above preparations from aminopolypeptidase by redissolving in water and reprecipitating with acetone (compare Grassmann and Klenk, 1930] resulted in almost complete destruction of both dipeptidase and aminopolypeptidase. A preparation (500 mg., containing 0·48 unit of dipeptidase and 0·20 unit of aminopolypeptidase per 10 mg.) was dissolved in water (50 cc.) and adjusted to p_H 7·0 with ammonia and cooled to 0°. Pure acetone (30 cc.) containing sufficient ammonia to keep the final mixture at p_H 7·0 was added, and the precipitate was centrifuged down and discarded. The centrifugate was treated with a further portion of acetone (25 cc.) at 0° and the precipitate was centrifuged down and dried by treatment with acetone and finally with ether. This material (150 mg.) contained 0·05 unit of dipeptidase and 0·02 unit of aminopolypeptidase per 10 mg.

An earlier method for the preparation of solutions free from "tryptic" activity [Grassmann and Haag, 1927] depended on the removal of the enzymic impurities by adsorption with a specially prepared suspension of aluminium hydroxide. This, when applied to the English top-yeast, gave a final solution which was free from proteinase but not from aminopolypeptidase.

The behaviour of a fresh yeast autolysate towards adsorption by aluminium hydroxide Cγ was investigated, in order to discover if a separation of dipeptidase and aminopolypeptidase could be effected by this means. English top-yeast (200 g.) was liquefied with ethyl acetate (20 cc.) and treated with water (220 cc.). Dilute ammonia was added to neutralise the acid as it was formed in the autolysis, and to maintain the p_H at 6·5. After 2 hours of this treatment

the yeast was centrifuged and carefully washed with water. It was now suspended in water (250 cc.) containing a few drops of toluene and allowed to autolyse for 17 hours, the p_H being maintained between 6·2 and 6·5. The yeast was centrifuged, and portions of 25 cc. each of the centrifugate were immediately cooled to 0°, adjusted to p_H 5·0 with *N* acetic acid, mixed with *M* sodium acetate-acetic acid buffer at p_H 5·0 (2 cc.) and treated in the following manner with a suspension of aluminium hydroxide Cγ (35 mg. Al_2O_3), the total volume being 35 cc. The adsorptions were carried out as quickly as possible and the temperature was kept below 10°. One portion was treated once with the adsorbent, another portion twice, and a third portion three times successively, the same amount of adsorbent being used in each case. The dipeptidase and aminopolypeptidase activities of the original autolysate and the three adsorbed portions were estimated (Table II).

Table II.

	Units of dipeptidase in solution representing 1 cc. of original autolysate	Units of aminopolypeptidase in solution representing 1 cc. of original autolysate
Original autolysate	3·35	1·80
After one adsorption	1·10	1·25
After two adsorptions	0·25	0·45
After three adsorptions	0·16	0·11

It is apparent from this experiment that simple adsorption with aluminium hydroxide Cγ under these conditions does not suffice for the separation of dipeptidase and aminopolypeptidase. Grassmann and Klenk [1930], using very similar conditions to those described above, observed that aminopolypeptidase was preferentially adsorbed, whereas in this experiment preferential adsorption of dipeptidase occurred. It would appear, therefore, that a quantitative difference exists between the adsorptive properties of the enzymes in autolysates of Löwenbräu yeast and English top-yeast.

Many attempts were made to obtain "pure" dipeptidase preparations. The following method, which is a modification of that of Grassmann and Klenk [1930], was found to be the most successful. English top-yeast (500 g.) was liquefied with ethyl acetate (50 cc.) and treated with water (500 cc.). 20 % ammonia was added carefully in order to maintain the suspension at p_H 6·5. After 2 hours the yeast was centrifuged and the dark brown centrifugate set aside. The yeast was carefully washed with water, again suspended in water (500 cc.) containing some toluene, allowed to autolyse for 20 hours at p_H 6·2–6·6 and room temperature and centrifuged off. Meantime an aluminium hydroxide suspension had been prepared [compare Grassmann and Haag, 1927]. A quantity (400 cc.) of the dark brown centrifugate which had been retained was diluted to 1600 cc. with water and adjusted to p_H 5·0 with *M* acetic acid. A suspension of aluminium hydroxide Cγ (700 mg. Al_2O_3) was added at 0° and after shaking the aluminium hydroxide was centrifuged down. This aluminium hydroxide which had thus adsorbed considerable amounts of autolysis products was now suspended in water and made up to 50 cc. Immediately after centrifuging from the yeast a portion of the autolysate (100 cc.) was adjusted to p_H 5 with acetic acid and mixed with *M* sodium acetate-acetic acid buffer at p_H 5·0 (2 cc.). This mixture was treated as quickly as possible four successive times at 0° with 10 cc. portions of the above aluminium hydroxide suspension. The residual enzyme solution

was then treated twice at 0° with successive portions (20 cc.) of a kaolin suspension (acid to phenolphthalein and alkaline to methyl red), containing 300 mg. of kaolin per cc. The kaolin was discarded, and a portion of this solution (120 cc.) was adjusted to p_H 7·0 with N ammonia, cooled to 0° and mixed with pure acetone (85 cc.) which contained sufficient ammonia to maintain the p_H at 7·0 (determined by previous trial on test portions). The resulting precipitate was centrifuged off and discarded. The centrifugate was treated with acetone (60 cc.) in the same way, and the precipitate was centrifuged and dried by washing three times with acetone, three times with ether and finally in a vacuum desiccator; yield 90 mg. This preparation contained 0·24 unit of dipeptidase and 0·005 unit of aminopolypeptidase per 10 mg. and was completely free from proteinase. After 2 months its activity remained unchanged.

This method differs from that of the German authors at several points. The most important deviation is in the larger volume of acetone used in precipitating the discarded solid of the first acetone treatment, and it is on this modification that the success of the method depends. The weight of the dry preparation and its enzymic content were very much less than those obtained by Grassmann and Klenk.

Aminopolypeptidase.

The method of Grassmann and Dyckerhoff [1928, 3] for the preparation of "pure" aminopolypeptidase from Löwenbräu yeast depends on the fact that the autolysis of the dead yeast cells in 0·3 % ammonia for 48 hours or less results in the liberation of all the proteinase activity and the destruction of all the dipeptidase activity. On the other hand, only part of the aminopolypeptidase is liberated during that time. Therefore, by centrifuging and washing the yeast after an autolysis lasting 48 hours, and by allowing it to autolyse in fresh water for a further 5 days, these authors obtained an autolysate containing aminopolypeptidase, but free from dipeptidase and proteinase. From this a dry preparation was obtained. On following this method with English top-yeast, the final preparation always contained traces of dipeptidase, but the modification described below yielded preparations free from both proteinase and dipeptidase.

English top-yeast (500 g.) was liquefied with chloroform (50 cc.) and treated with 0·35 % ammonia (500 cc.). After remaining at room temperature for 48 hours the yeast was centrifuged and thoroughly washed with water. It was then suspended in water (400 cc.) containing a little chloroform, and adjusted to p_H 7·0–7·2. After remaining for 5 days at room temperature the yeast was centrifuged off, and the centrifugate was treated with 2·5N acetic acid (65 cc.). The precipitate was centrifuged, suspended in water (20 cc.) and treated with drops of N ammonia until all but a trace of insoluble matter had dissolved (p_H 7·5). The solution, centrifuged from the solid, was dialysed for 14 hours in a cellophane bag against a slow stream of distilled water. The enzyme solution, cooled to 0°, was now added to three times its volume of pure acetone which had been cooled to − 15°. The temperature of the mixture was − 8°. The precipitate was centrifuged immediately and dried rapidly by washing three times with acetone, three times with ether and finally in a vacuum desiccator; yield 165 mg. This preparation contained 0·64 unit of aminopolypeptidase per 10 mg. and was completely free from dipeptidase and proteinase. When kept in a tightly stoppered tube its activity remained unaltered for 3 months.

Proteinase.

Grassmann and Dyckerhoff [1928, 3] obtained solutions of proteinase which were free from dipeptidase and aminopolypeptidase by making use of the following facts. Dipeptidase is destroyed during a preliminary autolysis of Löwenbräu yeast in 0·3 % ammonia lasting 17 hours. If the yeast be centrifuged, washed and allowed to autolyse in fresh water for a further 5 hours at p_H 5, the liberation of aminopolypeptidase is completely suppressed, whilst the proteinase enters the autolysate. The enzyme solution prepared in this way was adjusted to p_H 8·5 in order to precipitate inorganic phosphate, which was centrifuged off. After the reaction of the solution had been adjusted to p_H 5 the proteinase was adsorbed by treatment with aluminium hydroxide Cγ and eluted with di-ammonium phosphate. The German authors observed that the resulting proteinase solution underwent self-activation when preserved for 10 days at 0° and p_H 7·0, and they thus obtained a highly active proteinase solution, which was completely free from dipeptidase and aminopolypeptidase.

In the present investigation the following attempts were made to obtain pure proteinase preparations from English top-yeast and Dutch baker's yeast. In each case the final preparation was preserved at p_H 7·0 and 0° for 10 days before the enzymes were estimated (Table III) in order to allow the activator to develop.

(*a*) The method of Grassmann and Dyckerhoff [1928, 3] was carefully followed using English top-yeast. The purification by adsorption on aluminium hydroxide was carried out.

(*b*) As (*a*) using Dutch baker's yeast.

(*c*) The solution from (*b*) was adjusted to p_H 10 with 5N ammonia and maintained at that p_H at room temperature for 1 hour. It was then adjusted to p_H 7·0 with 5N acetic acid.

(*d*) As in (*c*), but the enzyme solution was maintained at p_H 10 for 3 hours.

(*e*) Using English top-yeast, the method of Grassmann and Dyckerhoff [1928, 3] was modified by carrying out the second autolysis at p_H 4·6 instead of 5·0.

(*f*) and (*g*). Procedure as in (*a*), except that the duration of the preliminary autolysis was reduced from 17 hours to 12 hours. In (*f*) the duration of the second autolysis was 3 hours, in (*g*) 5 hours.

(*h*) A portion (75 cc.) of the autolysate prepared from English top-yeast according to Grassmann and Dyckerhoff [1928, 3, p. 62] was treated with pure acetone (150 cc.) at room temperature. The precipitate was centrifuged off and redissolved in water (25 cc.). Acetone (30 cc.) was then added at room temperature, and the precipitate was centrifuged off and dried by washing with acetone, then ether and finally in a vacuum desiccator; yield 25 mg.

(*i*) A portion (75 cc.) of the same enzyme solution as that used in (*h*) was dialysed in a cellophane bag against running tap-water for 40 hours and then against a slow stream of distilled water for 6 hours. The solution was then mixed with acetone (100 cc.) at room temperature, and the precipitate was centrifuged off and dried as in (*h*); yield 35 mg.

The activities of the above preparation are recorded in Table III. All the preparations contained aminopolypeptidase and since that enzyme hydrolyses further the products of the proteinase hydrolysis of gelatin, no accurate figure for the increase in acidity due to the proteinase action could be obtained. However, the recorded increases in acidity give strong indication of the proteinase contents of the preparations. As already explained the figures for amino-

polypeptidase are also inaccurate in those preparations which contain dipeptidase. Complete hydrolysis of the *l*-leucylglycylglycine was obtained in the determination of the aminopolypeptidase activity of some of the preparations; this requires an increase in acidity of 1·00 cc. of *N*/20 KOH hydroxide.

Table III.

	Proteinase	Aminopolypeptidase	Dipeptidase
	In (*a*)–(*g*), 1 cc. of enzyme solution in a total volume of 10 cc. In (*h*) and (*i*), 10 mg. of dry preparation in 10 cc. Increase in acidity in 2 cc. portions after 24 hours. cc. *N*/20 KOH	In (*a*)–(*g*), 2 cc. of enzyme solution in a total volume of 10 cc. In (*h*) and (*i*), 20 mg. of dry preparation in 10 cc. Increase in acidity in 1 cc. portions after 24 hours. cc. *N*/20 KOH	In (*a*)–(*g*), 2 cc. of enzyme solution in a total volume of 10 cc. In (*h*) and (*i*), 20 mg. of dry preparation in 10 cc. Increase in acidity in 1 cc. portions after 24 hours. cc. *N*/20 KOH
(*a*)	1·10	1·01	0·05
(*b*)	1·60	0·96	0·09
(*c*)	1·33	0·83	0·01
(*d*)	0·97	0·56	0·00
(*e*)	0·46	0·88	Not determined
(*f*)	0·78	1·06	0·45
(*g*)	1·31	0·99	0·71
(*h*)	1·35	1·00	0·40
(*i*)	1·30	0·89	0·00

The results of these attempts to obtain a pure proteinase are here summarised. Using English top-yeast and Dutch baker's yeast the method of Grassmann and Dyckerhoff [1928, 3] yielded a preparation which contained considerable quantities of aminopolypeptidase and traces of dipeptidase (*a* and *b*). Treatment with ammonia readily destroyed these traces of dipeptidase, giving enzyme preparations which were completely free from dipeptidase but still contained aminopolypeptidase (*c* and *d*). Evidently in the case of these yeasts the liberation of aminopolypeptidase at p_H 5·0 is not completely suppressed; even at p_H 4·6 considerable quantities of aminopolypeptidase were liberated although less than half of the proteinase obtained at p_H 5·0 was set free (*e*).

Löwenbräu yeast liberates its proteinase only after about 16 hours' autolysis [Grassmann and Dyckerhoff, 1928, 3]. English top-yeast begins to liberate its proteinase much sooner, for a preliminary autolysis of 12 hours and a subsequent autolysis of 3 hours at p_H 5·0 yielded considerable quantities of the three proteolytic enzymes in the second autolysate (*f*). When the second autolysis was continued for 5 hours the amounts of the enzymes liberated had increased (*g*).

Grassmann and Dyckerhoff [1928, 1, 2] observed that precipitation of enzymic solutions with acetone at room temperature destroyed most of the aminopolypeptidase. In the case of the autolysates from English top-yeast, however, two successive precipitations did not destroy all the aminopolypeptidase activity. Moreover, some dipeptidase also persisted (*h*). Prolonged dialysis also destroyed aminopolypeptidase [Grassmann and Dyckerhoff, 1928, 1, 2]. Dialysis for 46 hours of the autolysate from English top-yeast followed by acetone precipitation yielded a dry preparation which still contained aminopolypeptidase but was free from dipeptidase (*i*).

Summary.

1. The liberation of the proteolytic enzymes, dipeptidase, aminopolypeptidase and proteinase by the autolysis of English top-yeast and Dutch baker's yeast has been studied and has been shown to differ in several respects from the

liberation of these enzymes from Löwenbräu yeast. Quantitative differences in the behaviour of the enzymes from these sources to adsorbents and other treatment have also been observed.

2. The methods used by Willstätter, Grassmann and their colleagues for the preparation of dipeptidase, aminopolypeptidase and proteinase from Löwenbräu yeast require modification when used for the preparation of these enzymes from English top-yeast.

3. By means of modifications of these methods, preparations of (*a*) dipeptidase completely free from proteinase and containing only traces of aminopolypeptidase, and (*b*) aminopolypeptidase free from both dipeptidase and proteinase have been obtained from English top-yeast.

4. Proteinase, free from dipeptidase but not from aminopolypeptidase, has been obtained from English top-yeast and Dutch baker's yeast.

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