RESEARCH PAPERS

PAPAIN : ASSAY PROCESS AND STANDARDS

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Introduction

In the course of work concerned with the preparation of sterile solutions of proteolytic enzymes for dental use it was necessary accurately to assay solutions of trypsin and of papain. The estimation of tryptic activity in pancreatin has been described in a previous paper[1]. The work on papain described below has followed a similar pattern. The assay processes of the 1934 and 1949 editions of the British Pharmaceutical Codex were tried first. The 1934 method was found to be so defective as to be unworkable. The 1949 process is better, but certain defects were discovered, the chief being the absence of activator in the digest. Investigation of this and other factors involved in the assay led to the establishment of an improved process. Finally the results of assaying a number of commercial samples of papain by the improved process and by the 1949 B.P.C. process were compared and suitable standards established.

Experimental

Choice of methods of assay. For the reasons given in the paper on trypsin[1] the method of assay chosen was formol titration to determine the extent of hydrolysis of casein used as substrate. In special investigations concerning the specific activities of purified papain, synthetic polypeptide substrates have been successfully used, but such substrates have nothing to recommend them when the object is to determine the general proteolytic capacity of crude papain.

Casein appears to have several advantages over gelatin as substrate particularly for use in conjunction with formol titrations. It contains a wider selection of amino-acids, being a complete protein, while gelatin is an incomplete protein. Roche and Mourge[2] have shown that papain liberates most of the valine and leucine from casein. Gelatin is deficient in these amino-acids. Greater ease of attack probably accounts for the advantage of casein over gelatin using formol titration as can be seen by calculating the formol titration alkali equivalent to 1 g. of papain. In the 1949 B.P.C. process 1 g. of papain acting upon 4 g. of gelatin for 3 hours causes 35 ml. of 0·1 N sodium hydroxide to be neutralised in the formol titration. In the process proposed, by similar calculation, 1 g. of papain acts upon 24 g. of casein for 30 minutes to give a formol titration of at least 180 ml. of 0·1 N sodium hydroxide. 1 to 4 is

much too high an enzyme substrate ratio to ensure complete saturation of the enzyme by the substrate. Even so, using gelatin, with 6 times as long a digestion period less than ¼ the amount of alkali is required as with a 1 to 24 enzyme ratio for casein. Some of the advantage of the new process is, of course, due to the digestion temperature of 60°C. replacing that of 37°C. For these reasons, except where mentioned, all the results recorded in this paper were obtained using casein as substrate.

Materials used. 5 samples of commercial papain were used. A was described as Ceylonese; B, C, D and E as South African. A and B were from the same supplier, B, C, D and E were from 4 different suppliers.

For reasons given in the paper[1] on pancreatin, B.D.H. "light white soluble casein" was used as substrate. It gave satisfactory repeatable results of the same values and degree of accuracy as those which were obtained by using the purified casein kindly supplied by Messrs. Bengers Food, Ltd. This latter complied with the B.P. specification for casein, but is not at present on the market.

Definition of terms. In a formol titration the solution may be adjusted to *p*H 7·0 to 8·7 (pink to phenolphthalein) by addition of alkali prior to the addition of formaldehyde. If carried out this is referred to as the "neutralising titration." After addition of formaldehyde alkali is added until the desired *p*H is obtained, usually 8·7. This constitutes the formol titration.

In an assay a blank is usually carried out using boiled enzyme. The difference between the formol titrations of the test and of the blank is called the "assay titration."

The "proposed process" refers to the assay process described on page 483 of this paper.

Activation. That papain is more active after the addition of an activator is an undisputed fact. Several substances can be used as activators; sodium sulphite, hydrogen sulphide, hydrogen cyanide, glutathione, and cysteine have all been used as well as other reducing agents[3]. Cysteine has the advantage of being easily available, convenient to handle, and non-poisonous. Further it has been shown by Bergman[4] that activation by cysteine produces an essentially greater enzymic activity than does activation with hydrogen cyanide, which is, in turn, more active than hydrogen sulphide[4]. For these reasons cysteine hydrochloride has been used in this work.

There are four principal theories regarding the mechanism of activation. 1. That papain is easily but reversibly inactivated by atmospheric oxidation and the activators establish reducing conditions[5]. 2. That the activators form a dissociable complex with the enzyme and thus function as co-enzymes[6]. 3. That papain is inactivated by traces of heavy metals which the activators remove by formation of complexes or insoluble compounds[7]. 4. That the activators protect and restore the -SH groups

which constitute an essential part of the active surface of the papain molecule[7]. These explanations are not mutually exclusive. The requisite -SH groups may be subject to oxidation by the atmosphere in the presence of traces of heavy metals acting as catalysts.

It might be argued that an activator should not be used in the assay process unless an activator is added to the pharmaceutical preparations of papain. It is, however, impossible to exclude activation. During the digestion process, and indeed in papain solutions on standing hydrogen sulphide is often generated, probably by decomposition of cysteine resulting from the hydrolysis of the protein of the substrate or of the proteins contained in the papain itself. This results in an unavoidable progressive activation which has been demonstrated by Gottschall[8] using meat as substrate.

Under these conditions the process can best be standardised by ensuring maximum activation during the whole digestion period. Table I shows the effect of increasing amounts of cysteine. 25 mg. per digest is sufficient for complete activation under the given conditions.

TABLE I

EFFECT OF INCREASING QUANTITIES OF CYSTEINE HYDROCHLORIDE ON THE ASSAY TITRATION. EACH DIGEST CONTAINED 25MG. OF PAPAIN AND WAS KEPT AT 60°C. FOR 30 MINUTES

Cysteine hydrochloride added per digest mg.	Assay titration. 0·1 N sodium hydroxide ml.
0	4·1
5	4·5
10	5·1
15	5·5
20	5·8
25	6·0
30	6·0
50	6·0

Willstatter, Grassman and Ambros[9] recommended incubation of the papain with the activator prior to commencing the digestion, suggesting that activation requires time. Experiments have shown that, provided that a quantity of activator capable of giving maximum activation is employed, the activity of the resulting enzyme-activator complex does not increase over a period of 0 to 5 hours at 37°C. After 24 hours the activity begins to fall. The activation appears to be almost instantaneous in the proposed process.

The marked effects of activation are shown in Table II, where the results given by 5 samples of papain without activation and with maximal activation are compared.

Temperature and pH. Figure 1 shows that the optimum temperature for the hydrolysis of casein by papain is about 78°C. At this point heat inactivation of the enzyme balances increase in reaction velocity with rise of temperature using a 30-minute digestion period. It is preferable to work at a temperature at which there is inappreciable heat inactivation of the enzyme during the digestion period. Table III shows the effect of preheating the papain and cysteine solution before carrying out the proposed assay process.

TABLE II

ACTIVITIES (ML. OF 0·1 N SODIUM HYDROXIDE) OF 5 SAMPLES OF PAPAIN USING THE PROPOSED PROCESS WITHOUT ACTIVATION AND WITH MAXIMAL ACTIVATION WITH CYSTEINE HYDROCHLORIDE

Papain sample	Without activation		With activation	
	Assay titration	Result per g. of papain	Assay titration	Result per g. of papain
A	1·5	60	4·5	180
B	4·0	160	6·2	248
C	3·6	144	5·9	236
D	3·2	128	5·4	216
E	4·6	184	6·0	240

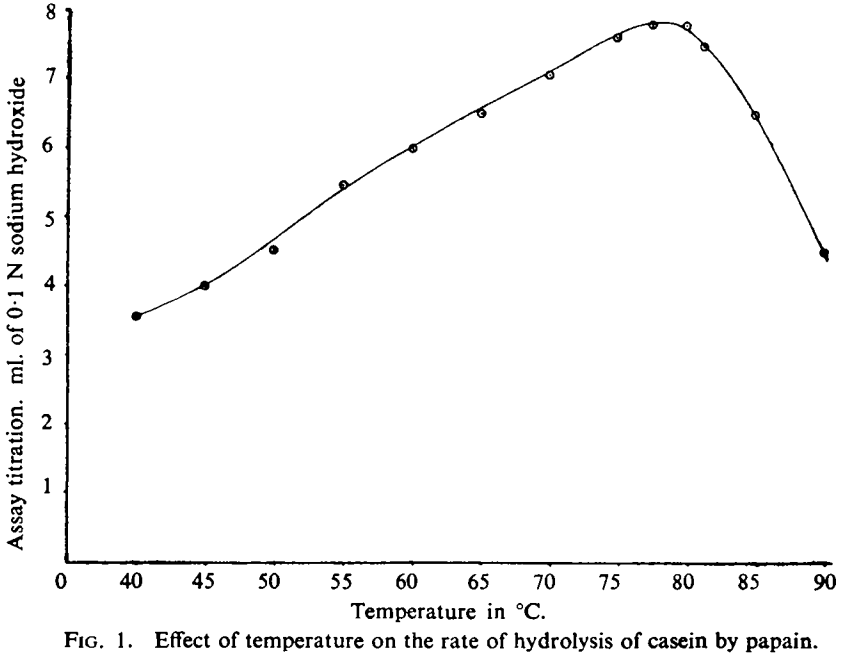

FIG. 1. Effect of temperature on the rate of hydrolysis of casein by papain.

Over 80°C. heat destruction is rapid and is complete in a few minutes at 100°C. 60°C. was chosen as the most suitable temperature for digestion since at this temperature there is no detectable inactivation in 30 minutes.

TABLE III

EFFECTS OF PREHEATING PAPAIN AND CYSTEINE SOLUTION ON THE ASSAY TITRATION

Temperature of preheating °C.	Time of preheating minutes	Assay titration
60	30	5·9
60	120	5·9
65	30	5·9
70	30	5·9
75	30	5·5
80	30	4·3

Greenberg and Winnick[10] found 7·0 to 7·5 to be the optimum *p*H for papain with ovalbumin, casein or denatured hæmoglobin as substrates. Hoover and Kokes[11] showed that the rate for the initial digestion of casein by papain was greatest at *p*H 7·0, although the final degree of hydrolysis was more extensive at *p*H 5·0. On this evidence 7·0 was chosen as the *p*H at which to start the digestion. Initially at 7·0, the *p*H falls to approximately 6·3 in a digest giving an assay titration of 6·0.

Kinetics of the hydrolysis of casein by papain. Figure 2 shows the

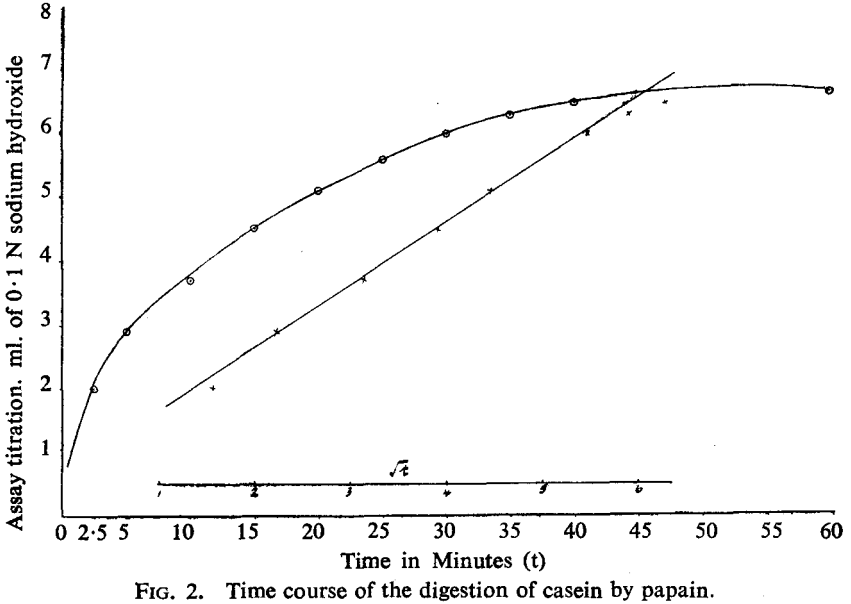

FIG. 2. Time course of the digestion of casein by papain.

time course of the digestion of casein by papain. Over the middle of the course a straight line is produced by plotting the figures for the assay titrations against the square root of the time. Figure 3 shows the relationship between enzyme concentration and the resultant assay titration. Except for low and high values, a straight line is produced by plotting the square root of the number of milligrams of papain in each digest

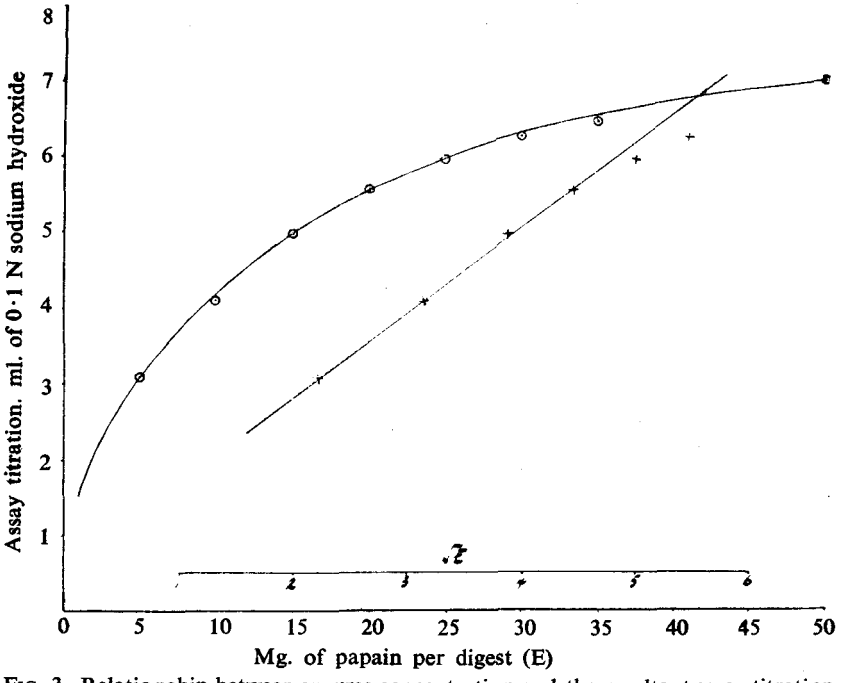

FIG. 3. Relationship between enzyme concentration and the resultant assay titration

against the resultant assay titration. In conjunction, the above findings indicate that, as with trypsin, the course of the digestion approximately follows Schutz's Law[12], at any rate over the mid-values. Schutz's Law states that $x = K\sqrt{E\,T}$ where T is the time, E the quantity of enzyme and x the resultant degree of digestion.

It follows that an assay process for papain should be devised so as to avoid the flat upper portion of the curve relating quantity of papain with assay titration, i.e., where there is little increase in the assay titration with further increase in the enzyme concentration. It is obvious that the assay cannot be devised so that there is a direct proportionality between the result and the quantity of enzyme present. If the object is to compare the activities of two or more samples of papain then it will be necessary first to prepare a curve relating enzyme quantity and assay titration under the given conditions. Figure 3 shows such a curve, and it will be seen that to increase the assay titration from 3·0 to 6·0 more than five times as much papain must be used.

Technique of the formol titration. At the completion of digestion,

for test and blank, the formol titration may be carried out in several ways; the different results are illustrated in Figure 4. There the number of mg. of papain used in each test is plotted against the resultant assay titration. The three different curves represent 4 different methods of

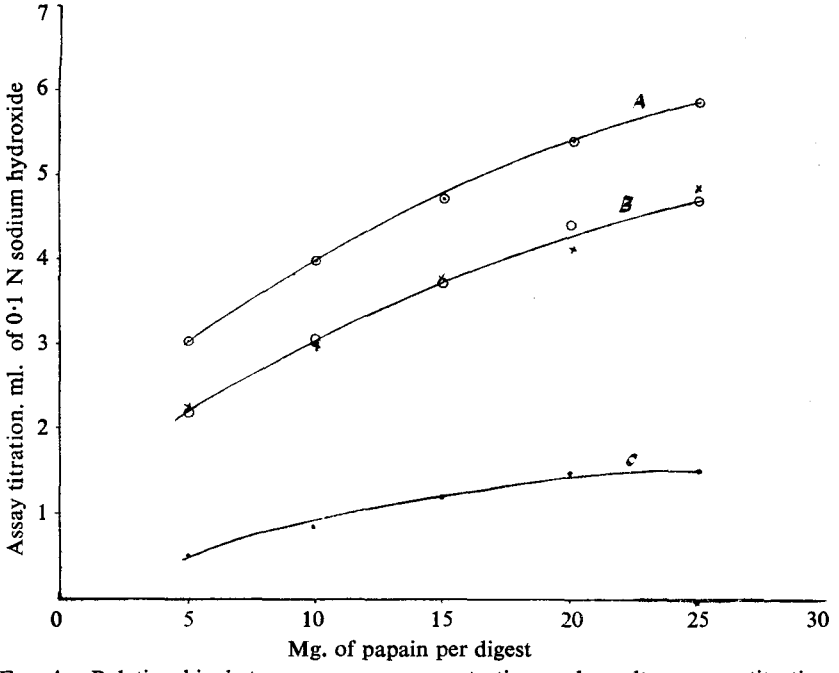

FIG. 4. Relationship between enzyme concentration and resultant assay titration using four different techniques of formol titration.

conducting the formol titration. In these experiments the glass electrode was used for all *p*H adjustments. To obtain curve A the neutralising titration was omitted and after addition of the solution of formaldehyde the mixture was titrated to *p*H 8·7, which is the same as pink to phenolphthalein.

Curve B (circles) was obtained by neutralising to *p*H 7·0, adding the solution of formaldehyde and titrating to *p*H 8·7. Curve B (crosses) was obtained by neutralising to *p*H 7·0, adding the solution of formaldehyde and titrating to *p*H 7·0. To understand why these two methods give the same curve it should be remembered that it is the assay titration which is plotted. The assay titration is the difference between the formol titrations of the test and the blank. It was found that in the 5 tests and in the 5 blanks the alkali required to raise the *p*H from 7·0 to 8·7 in the presence of formaldehyde was the same with an average value of 2·2 ml. of 0·1 N sodium hydroxide. Bringing the final *p*H to 8·7 instead of 7·0 simply means that 2·2 is added to both the test and blank, and when these latter are subtracted this, of course, cancels out. It is interesting to note that the Northrop[13] method of neutralising to *p*H 7·0, adding the formaldehyde and then titrating to 8·7, so useful in

the case of small quantities of pure amino-acids proves to be of no advantage when used on a digest resulting from the action of the crude papain extract on casein as substrate. This is the same conclusion as was reached with crude pancreatin acting upon casein.

Curve C was obtained by neutralising to *p*H 8·7 and titrating to 8·7 again, after addition of the solution of formaldehyde. This was the method recommended in the case of trypsin estimations, but there the digestion was commenced at *p*H 8·7. With papain the digestion commences at *p*H 7·0, and from Figure 4 it is obvious that the method giving curve A is the best. There is the greatest slope over the range 5 to 25 mg. of papain, and it is by far the simplest method. It was therefore adopted.

The Proposed Assay Process. Dissolve 4 g., accurately weighed, of B.D.H. light white soluble casein by shaking with 90 ml. of water. Adjust the *p*H to 7·0, using bromothymol blue as external indicator, and make up the volume to 100 ml. Dissolve 0·5 g., accurately weighed, of cysteine hydrochloride in 10 ml. of water and adjust the *p*H to 7·0. Triturate 0·5 g., accurately weighed, of papain with the solution of cysteine hydrochloride and make up to 100 ml.

In each of two flasks place 15 ml. of casein solution and 30 ml. of water, adjust the temperature to 60°C. and place in a water-bath at 60°C. To 1 flask add 5 ml. of the solution of papain and cysteine hydrochloride and to the other add 5 ml. of a portion of the same activated enzyme solution previously boiled and cooled. Maintain both at 60°C. for 30 minutes. Cool rapidly to room temperature and to each flask add 0·75 ml. of solution of phenolphthalein, 0·1 per cent. w/v, and 10 ml. of solution of formaldehyde previously neutralised to phenolphthalein. Titrate both liquids with 0·1N sodium hydroxide to a definite pink colour (*p*H 8·7). The difference between the two titrations should not be less than 4·5 ml. or more than 6·0 ml.

The Examination of Commercial Samples of Papain. As previously mentioned, 5 different samples of commercial papain were examined. The results obtained by the 1949 B.P.C. assay process are shown in Table IV, while those given by the proposed process are shown in Table V.

TABLE IV

RESULTS OBTAINED ON COMMERCIAL SAMPLES OF PAPAIN BY THE 1949 B.P.C. PROCESS ML. OF 0·1 N SODIUM HYDROXIDE

Sample	Formol titration of the blank	Formol titration of test	Assay titration	Result calculated for 1 g. of papain
A	8·0	14·0	6·0	33·7
B	11·6	23·6	12·0	67·4
C	10·8	20·4	9·6	54·0
D	12·4	21·6	9·2	51·7
E	12·0	22·5	10·5	59·0

TABLE V

RESULTS OBTAINED ON COMMERCIAL SAMPLES OF PAPAIN BY THE PROPOSED ASSAY PROCESS
ML. OF 0·1 N SODIUM HYDROXIDE

Sample	Formol titration blank	Formol titration test	Assay titration	Result calculated for 1 g. papain	Calculated for assay titration 4·5=100
A	5·5	10·0	4·5	180	100
B	5·8	12·0	6·2	248	236
C	5·6	11·5	5·9	236	196
D	5·6	11·0	5·4	216	156
E	5·8	11·8	6·0	240	208

Activation, used in the proposed process but omitted in the B.P.C. process, accounts for some of the differences and to assess this factor reference should be made to Table II.

After standing for 12 months at room temperature in the packets in which they were supplied 4 of the samples were reassayed by the proposed process. The results were identical with or differed by not more than 0·3 ml. from those obtained the previous year. 0·3 ml. is considered to be within the experimental error of the method.

DISCUSSION

As a result of using the higher temperature of 60°C. combined with maximal activation and casein as substrate it is possible to reduce the time for the assay of papain from 3 hours to 30 min. This is so even though the enzyme-casein ratio has the more satisfactory value of 1 to 24 instead of 1 to 4 as with gelatin as substrate.

The commercial samples of papain which were examined appeared to be satisfactory. They all passed the suggested minimum value of 4·5 for the assay titration in the new proposed process. The strongest was only twice as active as the weakest. This can be seen by reference to the last column of Table V where, using Figure 3 as reference curve, the results were calculated on the assumption that a papain giving an assay titration of 4·5 has an activity of 100. Using the B.P.C. process, sample A just failed to pass the minimum standard of 35 ml. for 1 g. of papain.

It is obvious that in adopting the new test and fixing the minimum value of the assay titration at 4·5 no significant alteration would be made in the standard required for papain.

SUMMARY

(1) Reasons are given for choosing formol titration with casein as substrate for the basis of the assay of papain.

(2) The importance of activation, preferably with cysteine hydrochloride, is established.

(3) A suggested assay process is described.

(4) The results of assaying 5 different commercial samples of papain both by the proposed process and by the 1949 B.P.C. process are recorded.

(5) Suitable standards are discussed.

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