Studies on the Preparation of Water-Insoluble Derivatives of Rennin and Chymotrypsin and their use in the Hydrolysis of Casein and the Clotting of Milk

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1. Enzymically active insoluble derivatives of chymotrypsin and rennin were prepared by coupling each enzyme to agarose as described by Porath, Axén & Ernback (1967) and rennin to aminoethylcellulose by the method of Habeeb (1967). 2. Agarose-chymotrypsin was stable over the range pH 2–9, but agarose-rennin released active enzyme into solution at above pH 2 and aminoethylcellulose-rennin was similarly unstable at certain pH values. 3. Each derivative appeared to catalyse the clotting of milk at 30°, but this was probably entirely due to enzyme released into solution from the carrier. 4. The presence of a competitive inhibitor of chymotrypsin during its coupling to agarose had no effect on the activity or stability of the resulting derivative. 5. The characteristics of agarose and cellulose render them not entirely suitable for use in a continuous system with milk.

Water-insoluble derivatives of enzymes may be prepared by either coupling the enzyme to an insoluble carrier or extensive cross-linking. The preparation, use and advantages of such derivatives have been reviewed by Silman & Katchalski (1966). In general the derivatives are less active than the corresponding soluble enzymes, particularly with high-molecular-weight substrates. However, this is offset by the fact that they can be used repeatedly since they can be recovered from a reaction mixture by filtration or centrifugation. In addition, they can be used for continuous processes in either packed beds or flow-through reactors (Lilly, Kay, Sharp & Wilson, 1968).

It was our aim in this work to determine whether insoluble enzymes could be used to catalyse the clotting of milk. Milk clotting occurs in two stages: first proteolysis takes place, followed by aggregation of the micelles to produce clotting (Jollès, 1966). These processes can be separated if the enzymic stage is carried out below 10°; the second stage then takes place on raising the temperature. The two stages occur simultaneously in conventional cheesemaking, but are usually separated in continuous processes such as the continuous curdmaker of Berridge (1968). The use of a reactor involving an insoluble enzyme for the enzymic stage of milk clotting would permit this to be carried out continuously. Such a reactor could easily be added to continuous curdmakers already in use and would also help to overcome the present world shortage of rennin (EC 3.4.4.3), the enzyme normally used

in cheesemaking. An additional advantage is that an insoluble enzyme would be separated from the milk before its conversion into cheese. This would enable a study of the role of rennin in the maturing of cheese to be made and also permit the use of different enzymes for clotting the milk and maturing the cheese.

In the present work various water-insoluble derivatives of proteolytic enzymes were prepared and their enzymic activities determined with protein and non-protein substrates. Each of these derivatives appeared to catalyse milk clotting, but this was probably entirely due to the release of soluble enzyme. This could not always be accounted for solely by the release of adsorbed enzyme; the evidence indicated the release of covalently bound material from agarose derivatives of rennin.

MATERIALS AND METHODS

The agarose used was Sepharose 2B obtained from Pharmacia (Uppsala, Sweden) and the AE-cellulose* was Whatman AE 11, a product of W. and R. Balston Ltd. (Maidstone, Kent). Crystalline α -chymotrypsin (EC 3.4.4.5) was obtained from Seravac Laboratories (Pty.) Ltd. (Maidenhead, Berks.). The crude rennet used was Chr. Hansen cheesemaking rennet, which was concentrated by dialysis against polyethylene glycol solution and then dialysed as required. Purified rennet was the precipitate obtained from crude rennet by saturation with NaCl (Berridge, 1955), dissolved in an appropriate buffer.

* Abbreviations: AE-cellulose, aminoethylcellulose; BPNE, *N*-benzoylphenylalanine β -naphthyl ester.

Crystalline rennin was prepared by Dr G. C. Cheeseman by the method of Berridge (1955).

Coupling of enzymes to insoluble carriers. Activation of agarose, coupling of chymotrypsin and washing of the product were carried out as described by Porath, Axén & Ernback (1967). Coupling of rennin to agarose was carried out by a modification of this procedure occasioned by the instability of rennin in alkaline solution. With the exception of derivative 6 (see Table 2), coupling was carried out in 0.1 M-Na₂HPO₄-KH₂PO₄ buffer, pH 6.3, and the product was washed for 24 hr. with each of 0.1 M-Na₂HPO₄-KH₂PO₄ buffer, pH 6.3, containing 1 M-NaCl, 0.01 M-HCl containing 1 M-NaCl, and 0.01 M-sodium acetate buffer, pH 4.1. It was stored in the last buffer at 4° in the presence of thymol. Derivative 6 was coupled in 0.1 M-NaHCO₃, but was washed in the same way as the other agarose-rennin derivatives. Controls, with agarose that had not been activated by treatment with CNBr, were carried out for both chymotrypsin and rennin to check the efficiency of the washing procedure in removing adsorbed enzyme.

Rennin was coupled to AE-cellulose by a modification of the method described by Habeeb (1967) for trypsin. A 50 mg. portion of purified rennet in 0.02 M-Na₂HPO₄-KH₂PO₄ buffer, pH 6.0, was added to 1 g. of AE-cellulose and mixed thoroughly. Then 0.1 ml. of 25% (w/v) glutaraldehyde previously brought to pH 6.0 was added and the mixture gently stirred overnight at 4°. A similar sample from which glutaraldehyde was omitted acted as the control. The mixture was then poured into a small column and washed as described for agarose-rennin. The product was stored at 4° in 0.01 M-sodium acetate buffer, pH 4.1, in the presence of thymol.

Determination of dry weights of insoluble enzyme suspensions and amount of enzyme bound. The specific activities of insoluble enzyme derivatives are expressed relative to the dry weight of the derivative rather than relative to the weight of enzyme bound. Dry weights were determined by evaporating a known volume of enzyme suspension to dryness at 90° and correcting for the dry weight of the suspending buffer. The amount of enzyme bound was determined from the nitrogen content of the derivative, corrected for that of the activated carrier, and the nitrogen content of the soluble enzyme. Nitrogen analyses were carried out by the micro-method of Lang (1958).

Enzyme-assay procedures. Chymotrypsin and chymotrypsin derivatives were assayed at 30° by either the casein-digestion method of Kunitz (1947) with total acid-precipitated casein as the substrate, or by a modification of the method of Ravin, Bernstein & Seligman (1954) with BPNE as substrate. In this latter method the enzyme was incubated at 30° for 20 min. with 0.3 mg. of BPNE in 0.1 M-tris-HCl buffer, pH 7.8, containing 5% (v/v) of ethanol in a total volume of 2 ml., and the liberated β -naphthol was determined spectrophotometrically at 526 nm. after coupling with tetra-azotized di-*o*-anisidine.

Rennin and rennin derivatives were assayed with crude κ -casein A as substrate. This was prepared by the method of Zittle & Custer (1963) with the omission of the ethanol-precipitation step. The enzyme was incubated at 30° with 0.5% (w/v) κ -casein in 0.1 M-sodium citrate buffer, pH 5.3, and the reaction stopped after 20 min. by the addition of trichloroacetic acid to a final concentration of 5% (w/v). After centrifugation, samples of the supernatant were taken for nitrogen determination (Lang, 1958).

RESULTS

Extent of coupling of enzymes to carriers and specific activities of derivatives with protein and low-molecular-weight substrates. Table 1 shows, for three different experiments, the extent of coupling of chymotrypsin to agarose under the conditions used by Porath *et al.* (1967) and the enzymic activities of the resulting derivatives. The results obtained with agarose that had not been treated with cyanogen bromide indicate that 95% of the enzyme was covalently bound in the derivative obtained from cyanogen bromide-treated agarose. Table 2 shows the corresponding results for various agarose-rennin derivatives. For rennin the coupling was normally carried out at a pH less than that used for chymotrypsin because rennin is unstable above pH 6.5. No rennin appeared to have been adsorbed on the agarose, therefore all the activity of the derivatives was presumably due to covalently bound enzyme. The enzymic activities of derivatives 1-4 were independent of the amount of enzyme bound. Derivatives 5 and 6 were of lower relative activity. With derivative 6 this is presumably because the coupling was carried out at a pH at which rennin is unstable, but the reason for the low activity of derivative 5 is not clear.

When purified rennet was coupled by means of glutaraldehyde to AE-cellulose the enzyme content of the derivative was 5.9% with a specific activity of 0.014% of that of soluble enzyme. The control material, without glutaraldehyde, contained no detectable enzyme on a nitrogen basis, but showed enzymic activity at 0.003% of that of soluble enzyme. Therefore it appeared that about 20% of the activity of the derivative was due to adsorbed rather than covalently bound enzyme and, in addition, the specific activity was lower than was normal for the corresponding agarose derivative.

Stability of insoluble enzyme derivatives with pH and time. Insoluble enzyme derivatives are valuable only if they can be both stored and used under conditions in which there is neither loss of enzymic activity nor release of activity in a soluble form. From the present point of view, pH and time appeared to be the most important variables.

After storage for 4 months at 4° in 0.01 M-acetate buffer, pH 4.1, a sample of agarose-chymotrypsin retained 82% of the original activity in a solid form with not more than 2% of the original activity present in solution. When agarose-chymotrypsin was stored for 8 days at 4° in 0.01 M solutions of buffers in the range pH 2-9, no detectable activity was released into solution in any case and the activity of the solid enzyme was significantly decreased at pH 2.0 (to 76% of the original) and at pH 6.2 (to 67% of the original), but not at pH 4.1, 7.8 and 9.0.

Table 1. *Coupling of chymotrypsin to agarose*

The methods used are described in the text. n.d., Not determined.

Derivative no.	Coupling conditions		% of enzyme in final material	Specific activity of total insoluble derivative (% of that of soluble enzyme)	
	Activated agarose (mg.)	Chymotrypsin (mg.)		BPNE as substrate	Casein as substrate
1	50	25	12.2	0.35	0.94
Control	50	25	0.3	0.007	n.d.
2	500	250	26.1	0.12	n.d.
3	50	25	17.4	0.15	3.4

Table 2. *Coupling of rennin to agarose*

The methods used are described in the text, the activities of the insoluble derivatives being assayed with κ -casein as substrate.

Derivative no.	Coupling conditions	% of enzyme in final material	Sp. activity of total insoluble derivative (% of that of soluble enzyme)
1	20 mg. of activated agarose, 27 mg. of crude rennet, pH 6.3	9.9	0.23
Control	20 mg. of non-activated agarose, 27 mg. of crude rennet, pH 6.3	0.0	0.00
2	500 mg. of activated agarose, 150 mg. of crude rennet, pH 6.3	2.3	0.15
3	50 mg. of activated agarose, 25 mg. of purified rennet, pH 6.3	2.8	0.15
4	200 mg. of activated agarose, 100 mg. of purified rennet, pH 6.3	15.0	0.17
5	40 mg. of activated agarose, 12.5 mg. of crystalline rennin, pH 6.3	4.4	0.018
6	40 mg. of activated agarose, 12.5 mg. of purified rennet, 0.1 M-NaHCO ₃	2.6	0.09

Table 3. *Stability of agarose-rennin with pH*

Samples (8 mg.) of agarose-rennin (derivative 3) were washed on sinters with the solutions indicated, then resuspended in 3 ml. of the same solutions and stored at 4° for 3 days. The mixtures were then filtered. The filtrates, diluted with 1 vol. of 0.05 M-acetate buffer, pH 5.3, and the solids, washed with 0.01 M-acetate buffer, pH 5.3, and resuspended in this buffer, were assayed with κ -casein as substrate.

Storage solution	Total activity (% of that of the final suspension of the sample stored at pH 5.4)		% of activity remaining in insoluble form
	Suspension	Filtrate	
0.01 M-HCl	79	4	95
0.01 M-Acetate buffer, pH 4.1	87	9	91
0.01 M-Acetate buffer, pH 5.4	100	43	70
0.01 M-Phosphate buffer, pH 6.2	91	178	34
0.01 M-Phosphate buffer, pH 7.0	89	236	27

The effects of storing agarose-rennin at various pH values are shown in Table 3. In contrast with agarose-chymotrypsin this derivative was unstable in the solid state, active enzyme being released into solution to an increasing extent as the pH was

raised. In addition the results indicate that the specific activity of the enzyme released into solution was greater than that of the original solid, since the total activity of the solid + filtrate increased with increase in the amount of enzyme released. This is

Table 4. *Stability of AE-cellulose- α -rennin with pH*

Samples (12.5 mg.) of AE-cellulose- α -rennin were treated as described in Table 3 for agarose- α -rennin except that 2 ml. of suspending solution was used and the mixtures were stored at 4° for 4 days.

Storage solution	Total activity (% of that of the final suspension of the sample stored at pH 6.2)		% of activity remaining in insoluble form
	Suspension	Filtrate	
0.01 M-HCl	13	80	14
0.01 M-Acetate buffer, pH 4.1	16	0	100
0.01 M-Acetate buffer, pH 5.4	65	5	93
0.01 M-Phosphate buffer, pH 6.2	100	3	97
0.01 M-Phosphate buffer, pH 7.0	60	22	73

Fig. 1. Time-course of release of soluble enzyme from agarose- α -rennin at pH 6.2. A portion (16.5 mg.) of agarose- α -rennin (derivative 4) was washed on a sinter with 0.01 M-phosphate buffer, pH 6.2, and then resuspended in 15 ml. of this buffer. The mixture was stirred at 4°. At intervals thereafter samples were filtered and the solid material and filtrate were recovered and assayed as described for Table 3. ●, Activity of solid material; ○, activity of filtrate.

confirmed by Fig. 1, which shows the time-course of the release of soluble enzyme from agarose- α -rennin (derivative 4) at pH 6.2. With derivatives prepared from crystalline rennin (derivative 5) and from purified rennet at high pH (derivative 6) the proportion of enzymic activity released into solution was decreased to about 55% of that with derivative 4. This may be related to their relatively lower activity, or to stronger binding of the enzyme to the carrier.

The effects of incubating AE-cellulose at 4° at various pH values is shown in Table 4. In this case the insoluble enzyme was maximally stable with respect to both release of enzymic activity into solution and activity of the solid at slightly acid pH.

Stability of insoluble derivatives during enzymic assays. Results detailed in the previous section show that both agarose- α -rennin and AE-cellulose- α -rennin release enzymic activity into solution under certain conditions, and results described below show that the same is true for agarose-chymotrypsin. Therefore the question arose whether the enzymic activities determined by the assay procedures were entirely due to the solid enzymes or were partly or completely due to enzymes released into solution during the assay. This was answered by carrying out the assay of each insoluble enzyme by the standard procedure except that, at the end of the normal 20 min. incubation period, the insoluble enzyme was rapidly filtered off and the filtrate divided into two portions. The first portion was immediately acidified but the second was incubated for a further 20 min. at 30° before acidification. The amount of hydrolysis of substrate during the second incubation period provided a measure of the amount of enzyme in solution at the end of the first period. Therefore the ratio of the amount of hydrolysis in the second to that in the first period gave the maximal contribution of soluble enzyme to the total enzymic activity.

On this basis, about 5% of the activity of agarose-chymotrypsin was due to soluble enzyme when assayed with either whole casein or BPNE as substrate. With agarose- α -rennin (derivatives 5 and 6) and AE-cellulose- α -rennin no perceptible soluble enzyme was released. With the control AE-cellulose- α -rennin, where glutaraldehyde was absent during the preparation, 16% of the total activity was due to enzyme released into solution.

Clotting of milk by insoluble enzyme derivatives. Each of the three types of insoluble enzyme derivatives prepared apparently catalysed the clotting of milk at 30°, but in all cases this was shown to be almost entirely due to released soluble enzyme. The results are shown in Table 5, the activities of the insoluble derivatives being corrected for soluble enzyme. The two sets of results

Table 5. *Clotting of milk by insoluble enzyme derivatives at 30°*

In each case a sample of the enzyme derivative was washed thoroughly on a sinter with separated milk at pH 6.6 and then resuspended in water. Then 0.2 ml. of the suspension was added to 0.5 ml. of milk and the mixture shaken at 30° until the milk clotted. The mixture was filtered on a sinter, a 0.2 ml. sample of the filtrate added to a further 0.5 ml. of milk and the clotting time determined. The first clotting time, recorded below, was a measure of the total enzyme activity of the derivative due to both soluble and insoluble enzyme. The second clotting time enabled calculation of the maximal contribution of soluble enzyme to the activity.

Enzyme	Enzyme added (mg./0.5 ml. of milk)	Clotting time (min.)	% of activity due to released soluble enzyme (maximum)	Sp. activity of total insoluble derivative (% of that of the soluble enzyme)
Agarose- <i>rennin</i> (derivative 5)	1.2	51	12-60	0.55×10^{-3} - 1.21×10^{-3}
	1.2	81	100+	0
Agarose- <i>rennin</i> (derivative 6)	1.7	115	100+	0
	1.7	142	100+	0
Agarose-chymotrypsin (derivative 2)	5.0	330-400	100+	0
Agarose-chymotrypsin (derivative 3)	2.1	480-600	100+	0
AE-cellulose- <i>rennin</i>	6.2	127	100+	0
Control AE-cellulose- <i>rennin</i>	4.4	100	100+	0

Fig. 2. Release of soluble enzyme from agarose-*rennin* derivatives on incubation with milk. The experiment was carried out as described for Table 5 except that samples of enzyme-milk mixture were shaken at 30° for the times indicated before filtration. ●, Derivative 5; ○, derivative 6.

given for each of the agarose-*rennin* derivatives represent two different experiments, the second being carried out 3 months after the first. The range given for the clotting times of the agarose-chymotrypsin derivatives arises from the difficulty of recording a definite clotting time, since the clot dissolves soon after formation owing to further hydrolysis by the enzyme.

At least part of the enzymic activity of each derivative was due to released soluble enzyme, the proportion being calculated from the activity in the filtrate after clotting had occurred. This is therefore a maximum value, which is equal to the true

value only if all the soluble enzyme is released at the beginning of the incubation period. A true value can be obtained from the time-course of the release of soluble enzyme. As shown in Fig. 2, the release of soluble enzyme from each of the agarose-*rennin* derivatives into milk was approximately linear with time after a rapid initial rise. The enzymic activity in solution at half the clotting time was equal to the average soluble activity present during the incubation of the agarose-*rennin* derivative with milk. From this it was calculated that the entire activity of each derivative could be accounted for by released soluble enzyme. The time-course suggests that at least most of the enzyme released into solution was originally covalently bound. This release appears to be mainly a pH effect (cf. Fig. 1): on assay with κ -casein at the pH of milk up to 90% and 58% respectively of the activities of derivatives 5 and 6 were due to soluble enzyme.

Detailed studies of the release of soluble enzyme from agarose-chymotrypsin and AE-cellulose-*rennin* derivatives were not carried out. However, it was observed that, even after extensive washing of agarose-chymotrypsin with milk, the release of soluble enzyme reached its maximum within 1 min. of suspending the derivative in milk at 4°. This suggests that the released enzyme had previously been adsorbed on the agarose rather than covalently bound and this probably accounted for the entire activity of the derivative. With AE-cellulose-*rennin* the control derivative had the greater milk-clotting activity, suggesting that all the activity of the derivative in which *rennin* was covalently bound was due to adsorbed enzyme.

Fig. 3. Hydrolysis of total casein and κ -casein by columns of agarose-chymotrypsin and agarose-rennin at 4°. For the agarose-chymotrypsin column, 13.6mg. of agarose-chymotrypsin (derivative 1) was poured into a 1.1cm.-diameter column and washed with 50ml. of 0.1M-tris-HCl buffer, pH 7.8. Then 0.5% total casein in 0.1M-tris-HCl buffer, pH 7.8, was passed through the column at various flow rates. Samples of the effluent were collected and the extent of hydrolysis was determined from the extinction at 280nm. of the supernatants after acidification with 3% (w/v) trichloroacetic acid. This was related to the extinction at 280nm. after hydrolysis with crystalline chymotrypsin. The agarose-rennin column was set up similarly except that 8.0mg. of agarose-rennin was used, the buffer was 0.1M-citrate, pH 5.3, and the substrate was 0.5% κ -casein. The extent of hydrolysis in samples of the effluent was determined from the nitrogen content of the supernatants after acidification with 5% (w/v) trichloroacetic acid and related to the nitrogen content after maximal hydrolysis with crude rennet. ●, Agarose-chymotrypsin column; ○, agarose-rennin column.

Competitive inhibition of chymotrypsin during the coupling reaction. The binding of an enzyme to an insoluble carrier presumably involves several different covalent bonds and thus might decrease the ease with which conformational changes occur. There is evidence that this occurs in that certain insoluble derivatives are more stable than the corresponding soluble enzymes to heat and denaturing agents such as urea (Silman & Katchalski, 1966; Hornby, Lilly & Crook, 1966; Wilson, Kay & Lilly, 1968). Therefore conformational changes during enzyme action (Koshland, 1960) might be impeded by binding of the enzyme to the carrier. Thus the presence of a competitive inhibitor during coupling, which would hold the enzyme in its active conformational state, might both increase the activity of the bound enzyme and decrease any tendency to solubilize. This was investigated for agarose-chymotrypsin by using 0.05 M-DL-N-acetyltryptophan as the competitive inhibitor (Huang & Niemann, 1951); this was present during coupling but was subsequently washed out of the derivative. It had no significant effect on either the enzymic

activity or the extent of solubilization of the preparation with BPNE, casein or milk.

Use of insoluble enzyme derivatives in a continuous system. Lilly *et al.* (1968) have pointed out that insoluble enzymes can be used continuously in either packed beds or flow-through reactors. The applicability of these for use with protein substrates and milk has been investigated with agarose-chymotrypsin and agarose-rennin derivatives.

The hydrolysis of total casein and κ -casein by passage through columns of agarose-chymotrypsin and agarose-rennin respectively at 4° is shown in Fig. 3. In each case 250ml. of substrate solution was passed through the column over a period of 3 days, the flow rates being varied randomly. The enzymic activity did not change significantly over the period of either experiment. The volume of the agarose-chymotrypsin column was about 1ml., so, assuming that the void volume was not greatly different, maximal hydrolysis was obtained with a contact time between substrate and enzyme of about 20min. and 50% hydrolysis required a contact time of about 1.7min. Since the volume of the agarose-rennin column was about 0.5ml., complete hydrolysis required an enzyme-substrate contact time of about 62min. and 50% hydrolysis about 12min.

Attempts to pass milk through columns were less satisfactory as the column eventually clogged, particularly at low flow rates. Therefore the use of a flow-through reactor was tried, but this was not successful as the agarose beads quickly broke up as a result of the vigorous stirring.

DISCUSSION

By using slight modifications of the methods of Porath *et al.* (1967) and Habeeb (1967) insoluble derivatives of rennin that catalyse the hydrolysis of κ -casein have been prepared. In certain cases they may also catalyse the clotting of milk, but most of their activity in this respect was due to released soluble enzyme. While the present work was in progress, Jansen & Olson (1969) reported the clotting of milk by an insoluble papain derivative.

The specific activities of the bound enzymes were never more than 20% of those of the corresponding enzymes in solution. This is common to most insoluble enzyme derivatives and is usually attributed to steric hindrance by the carrier to the approach of the substrate (Silman & Katchalski, 1966). This would be more serious as the size of the substrate increases and explains the very low or zero activity of the enzyme derivatives on casein micelles in milk (particle wt. 10^7 – 328×10^7 ; Nitschmann, 1949), compared with those on

solutions of total casein (particle wt. approx. 100 000; Svedberg, Carpenter & Carpenter, 1930) and κ -casein (particle wt. approx. 325 000; estimated from the sedimentation coefficient determined by Swaisgood & Brunner, 1962). A second manifestation of steric hindrance can be deduced from the results in Table 2 relating to agarose-*rennin* derivatives 1-4. This is the approximately constant enzymic activity of the derivative regardless of the amount of enzyme bound. A similar phenomenon was observed when ficin was bound to CM-cellulose (Hornby *et al.* 1966). It would appear that the first enzyme molecules are bound in the most accessible parts of the carrier and show most of the activity and that the excess, bound in less accessible areas, is almost inactive.

The relatively low specific activities of insoluble derivatives increase the effects of contamination by soluble enzyme. This can result either from adsorption to the carrier as appeared to be the case with AE-cellulose-*rennin* and agarose-chymotrypsin or from release of apparently covalently bound enzyme into solution as was observed with agarose-*rennin* (these conclusions are drawn from the data on the relative activities of derivatives prepared from activated and non-activated carriers, the conditions under which soluble enzymes were released and the time-courses of release of soluble enzymes into milk). The specific activities of enzymes released into solution from the bound form were not determined in this work, but it is clear (e.g. from Table 3 and Fig. 1) that they were considerably greater than those of the corresponding bound enzymes. In fact, a large amount of soluble enzyme, in terms of activity, could be released without any apparent change in the activity of the solid. This casts doubt on one criterion of insoluble-enzyme stability, namely constant specific activity of the solid derivative used in some work (Axén & Porath, 1966; Habeeb, 1967; Porath *et al.* 1967; Ogata, Ottesen & Svendsen, 1968), and emphasizes the greater importance of determining any release of activity into solution.

The results reported here also indicate the necessity of determining any release of soluble activity during the enzymic assay (Bar-Eli & Katchalski, 1963; Riesel & Katchalski, 1964). This is particularly important with large substrates because of the relatively low specific activity of the insoluble material. In this respect the use of milk creates a particular problem. It has not been possible to conclude certainly whether or not the insoluble enzyme derivatives used here showed any activity on milk because of the relatively high activity of the released soluble enzyme.

It was expected that the presence of a competitive inhibitor during coupling would increase both the activity and the stability of the resulting derivative.

The enzyme molecules would presumably be mainly present in the active conformation and would be expected to couple in this form. Once bound to the carrier they would be held in the active conformation after the competitive inhibitor had been washed out. The lack of effect of the competitive inhibitor may indicate that either chymotrypsin alters its conformation to only a small extent on interaction with the substrate or the binding of the enzyme to the carrier is not so rigid as to impede conformational changes.

The aim of the work was not achieved in that a totally insoluble enzyme derivative suitable for use in milk clotting was not prepared. In this respect the most encouraging results were obtained with agarose-*rennin* derivatives. However, agarose does not appear suitable for use in continuous processes involving milk. It cannot be used in either a stirred reactor, since the beads rapidly break up on stirring, or in a packed bed, because particles from the milk eventually block the column. Cellulose or its derivatives are also not entirely suitable because of the difficulty of completely removing adsorbed enzyme (Hornby *et al.* 1966; Whittam, Edwards & Wheeler, 1968).

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REFERENCES

- Axén, R. & Porath, J. (1966). *Nature, Lond.*, **210**, 367.
- Bar-Eli, A. & Katchalski, E. (1963). *J. biol. Chem.* **238**, 1690.
- Berridge, N. J. (1955). In *Methods in Enzymology*, vol. 2, p. 69. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Berridge, N. J. (1968). *J. Soc. Dairy Technol.* **21**, 52.
- Habeeb, A. F. S. A. (1967). *Arch. Biochem. Biophys.* **119**, 264.
- Hornby, W. E., Lilly, M. D. & Crook, E. M. (1966). *Biochem. J.* **98**, 420.
- Huang, H. T. & Niemann, C. (1951). *J. Amer. chem. Soc.* **73**, 3223.
- Jansen, E. F. & Olson, A. C. (1969). *Arch. Biochem. Biophys.* **129**, 221.
- Jollès, P. (1966). *Angew. Chem. (int. Ed.)*, **5**, 558.
- Koshland, D. E. (1960). *Advanc. Enzymol.* **22**, 45.
- Kunitz, M. (1947). *J. gen. Physiol.* **30**, 291.
- Lang, C. A. (1958). *Analyt. Chem.* **30**, 1692.
- Lilly, M. D., Kay, G., Sharp, A. K. & Wilson, R. J. H. (1968). *Biochem. J.* **107**, 5P.
- Nitschmann, H. (1949). *Helv. chim. acta*, **32**, 1258.
- Ogata, K., Ottesen, M. & Svendsen, I. (1968). *Biochim. biophys. Acta*, **159**, 403.
- Porath, J., Axén, R. & Ernback, S. (1967). *Nature, Lond.*, **215**, 1491.
- Ravin, H. A., Bernstein, P. & Seligman, A. M. (1954). *J. biol. Chem.* **208**, 1.
- Riesel, E. & Katchalski, E. (1964). *J. biol. Chem.* **239**, 1521.

- Silman, I. H. & Katchalski, E. (1966). *Annu. Rev. Biochem.* **35**, 873.
- Svedberg, T., Carpenter, L. M. & Carpenter, D. C. (1930). *J. Amer. chem. Soc.* **52**, 701.
- Swaisgood, H. E. & Brunner, J. R. (1962). *J. Dairy Sci.* **45**, 1.
- Whittam, R., Edwards, B. A. & Wheeler, K. P. (1968). *Biochem. J.* **107**, 3 p.
- Wilson, R. J. H., Kay, G. & Lilly, M. D. (1968). *Biochem. J.* **108**, 845.
- Zittle, C. A. & Custer, J. H. (1963). *J. Dairy Sci.* **46**, 1183.