Activity and Specificity of Rennin

ALTHOUGH proteolytic activity has been shown by Berridge[1] in a partially crystalline preparation of rennin, and by De Baun, Connors and Sullivan[2] in a preparation 75 per cent pure, there has hitherto been no published proof that the activity is not due to contamination. Moreover, it has been implied that rennin has only weak proteolytic activity[3].

Crystalline rennin has been prepared by the method of Berridge and Woodward[4] from Benger's commercial rennet. Evidence has now been obtained that it is a powerful proteolytic enzyme with a specificity similar to that of pepsin. Proteolytic activity was measured on denatured hæmoglobin by the method of Anson as described by Northrop, Kunitz and Herriott[5], and milk-clotting activity was measured by the technique of Berridge. Throughout the purification, the ratio of these two activities remained constant. This applied even to the later supernatants from salting out with sodium chloride. Recrystallization of rennin has no significant effect on its activity.

	P.A.	*R.*
1st crystallization	0·104	2·33
Mother liquor from 1st crystallization	0·076	2·37
2nd crystallization	0·108	2·42
Mother liquor from 2nd crystallization	0·118	2·44
3rd crystallization	0·113	2·27

P.A.: Proteolytic activity per mgm. of total nitrogen in units exactly as defined in ref. 5.
R: Ratio of milk-clotting activity (in units defined by Berridge) to proteolytic activity.

It can be seen that, measured in this way, rennin has a proteolytic activity similar to that of trypsin and quite comparable with that of the other endopeptidases.

The *B* chain of insulin was considered to be a good substrate for an initial study of the proteolytic specificity of rennin. In most experiments, 1·5 mgm./ml. of the *B* chain was incubated overnight at 30° C. with 0·03 mgm./ml. of twice-crystallized rennin. The digest was then freeze-dried and subjected to ionophoresis on Whatman No. 4 paper at *p*H 6·5. The peptide bands were eluted and the eluates hydrolysed with 20 per cent hydrochloric acid, using the techniques of Sanger and his colleagues[6]. The hydrochloric acid was removed and the constituent amino-acids of the separated peptides were identified by one-dimensional chromatography with phenol/ammonia and also *sec*-butanol/formic acid solvent mixtures. The results indicate that rennin splits the *B* chain of insulin in the following positions:

Phe. Val. Asp. Glu. His. Leu. $CySO_3$. Gly. Ser. His. Leu. ↓ Val. Glu.

Ala. Leu. ↓ Tyr. ↓ Leu. Val. $CySO_3$. Gly. Glu. Arg. Phe. ↓ Phe. ↓

Tyr. Thr. Pro. Lys. Ala.

These are all bonds which are split by pepsin. In contrast to chymotrypsin, which also splits some of these bonds, rennin does not liberate the peptide Thr. Pro. Lys. Ala. from the *B* chain, neither does it act on the chymotrypsin substrate acetyl-tyrosine ethyl ester. However, although the *B* chain digestion by rennin has been repeated several times, no evidence has been found for the breakage of additional bonds which Sanger and his colleagues have found to be split by pepsin. This slightly narrower specificity of rennin is confirmed by comparisons between rennin digests of the *B* chain with pepsin digests. Another difference is that a rennin digestion always leaves equal quantities of the peptides Phe. Tyr. Thr. Pro. Lys. Ala. and Tyr. Thr. Pro. Lys. Ala., whereas pepsin may leave only a trace of the former peptide. Experiments on the progress of the digestion indicate that rennin splits bond 1 more slowly than bonds 2, 3, 4, and 5.

Since pepsin has been shown to be present in commercial rennet, it is important to eliminate any possibility of pepsin contamination interfering with specificity experiments. The presence of 5 per cent of pepsin can be quantitatively detected by electrophoresis. A 0·8 per cent solution of twice-crystallized rennin showed a single symmetrical peak during Tiselius electrophoresis at *p*H 6·5. 5 per cent of crystalline pepsin was added to an identical solution and another electrophoresis was performed. After half an hour a small peak with 5 per cent of the area of the main peak separated. A 4 hr. rennin digest of the *B* chain was then compared with a digest obtained under identical conditions but using a pepsin solution 5 per cent the strength of the rennin solution. Splitting of the *B* chain was considerable in the rennin digest but was negligible in the pepsin digest.

The fact that the action on the *B* chain is not due to pepsin has been further shown by quantitative experiments on the *B* chain using the ninhydrin method of Moore and Stein for following the digestion. Pepsin has an optimum for effect on the *B* chain of about *p*H 2, whereas that of rennin is *p*H 4.

It has been shown chromatographically that rennin at *p*H 6 splits the pepsin substrate carbobenzoxy-glutamyltyrosine. As with pepsin, long incubation periods and high concentrations of enzyme are required.

In conclusion, it must be noted that Holter and Li[7] found that crystalline rennin prepared by Berridge[1] had phosphoamidase activity on the substrate N-(*p*-chlorophenyl)-amidophosphoric acid. Attempts to confirm this on a substrate supplied by Light and Co., Ltd. ('*p*-chloranilidophosphonic acid (Phosphoamidase)'), have failed. Although activity was present in the commercial rennet (measured by the liberation of phosphate as described by Holter and Li), no activity could be detected in crystalline rennin. The substrate gave a high blank. It is suggested that the phosphoamidase activity is due to a contaminant which is not present in crystals prepared by Berridge's latest method.

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