The Disulphide Bridges and Soluble Tryptic Peptides of Calf Rennin

By B. FOLTMANN AND B. S. HARTLEY

Medical Research Council Laboratory of Molecular Biology, Cambridge

(Received 21 February 1967)

1. Cysteic acid peptides from various digests of calf rennin were purified by diagonal paper electrophoresis. 2. The amino acid sequence of these peptides accounts for 38 amino acids around three unique disulphide bridges in rennin. 3. One bridge connects two acidic regions of the chain, one forms a loop of five residues and the other a loop of six residues. 4. These bridges are homologous with those of hog pepsin. 5. Tryptic peptides from the *C*-terminus of rennin account for 22 residues, 17 of which are homologous with the *C*-terminus of pepsin. 6. Altogether, sequences accounting for 94 of the 270 residues in rennin are described and the degree of homology with pepsin approximates to 70%.

Calf rennin (EC 3.4.4.3) and hog pepsin (EC 3.4.4.1) are very similar in proteolytic activity (Bang-Jensen, Foltmann & Rombauts, 1964), and the amino acid sequences of these gastric enzymes may show common features such as have been observed with the pancreatic proteinases (Hartley, Brown, Kauffman & Smillie, 1965). Further, pepsin and rennin are relatively easy to crystallize and have begun to interest X-ray crystallographers. These reasons encouraged us to start sequence studies on crystalline rennin.

Crystalline rennin consists of two chromatographically separable components, rennin A and rennin B, which can be respectively obtained by activation of two separable zymogens, prorennin A and prorennin B (Foltmann, 1962). It is therefore possible that crystalline rennin is a mixture of two isoenzymes with different amino acid sequences. However, Foltmann (1964) could detect no significant differences between the amino acid compositions of purified prorennin B and total prorennin or purified rennin B and total crystalline rennin. Therefore for these preliminary studies we have used crystalline rennin rather than the purified components, in the hope that any sequence differences would appear during the work.

As a first approach to determining the degree of homology between pepsin and rennin, we have studied the sequences around the three disulphide bridges, since parallel studies of the disulphide bridges of pepsin have been carried out in this Laboratory (J. Tang & B. S. Hartley, unpublished work). Studies of the soluble tryptic peptides have also revealed a high degree of homology in the *C*-terminal sequences of pepsin (Dopheide, Moore & Stein, 1967) and rennin. A preliminary report of this work has been given by Foltmann (1966).

MATERIALS

Crystalline rennin. This was prepared from commercial rennet powder (Chr. Hansen's Laboratory, Copenhagen, Denmark) and crystallized as described by Foltmann (1959).

Other enzymes. Trypsin, α -chymotrypsin, carboxypeptidase A, pepsin and papain were crystalline products from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Crystalline subtilisin B was from Novo Terapeutisk Laboratorium, Copenhagen, Denmark.

METHODS

Rennin digestion of denatured rennin. Crystalline rennin (280 mg.) was dissolved by dialysis against distilled water, and 8 ml. of this solution containing 200 mg. of rennin was boiled for 15 min. to denature the enzyme. This denatured sample was then diluted with 8 ml. of 0.1 M-pyridine-acetate buffer, pH 3.5, and stirred at room temperature with 25 mg. of the remaining active rennin. The considerable amount of precipitate that remained even after 20 hr. digestion was spun down, resuspended in pH 3.5 buffer and digested again for 24 hr. with a second 25 mg. portion of rennin. The precipitate that remained was digested a third time with 25 mg. of rennin and the supernatants from all three digests were pooled.

Pepsin digestion of the rennin 'core'. The insoluble precipitate that remained after the rennin digestion was suspended in 10 ml. of 5% (v/v) formic acid and digested with 10 mg. of pepsin at room temperature overnight with stirring. Negligible precipitate remained after this treatment.

Chymotrypsin digestion of denatured rennin. Crystalline rennin (140 mg.) was dissolved by dialysis against distilled water and diluted to 8 ml. in the reaction vessel of a pH-stat. The solution was adjusted rapidly to pH 10.6 with 0.1 N-NaOH and incubated for 30 min. at room temperature to denature the rennin. The pH was then adjusted to 8.5 with HCl and 2 ml. of a solution containing α -chymotrypsin (1 mg./ml.) and soya-bean trypsin inhibitor (1 mg./ml.) was added. The solution became turbid after 2 hr. digestion, and after 5 hr. digestion the alkali uptake suggested that about

20 bonds/mol. of rennin had been split. The pH was then adjusted to 6.5 with HCl and the insoluble precipitate was spun off.

Pepsin digestion of the chymotryptic 'core'. This insoluble 'core' was freeze-dried, dissolved in 0.5 ml. of 99% (v/v) formic acid and diluted immediately into 5 ml. of water containing 5 mg. of pepsin. A fine precipitate appeared that remained even after 16 hr. digestion with stirring at room temperature. The remaining precipitate was spun off, and the supernatant peptides were examined by the diagonal paper-electrophoretic procedure.

Diagonal paper electrophoresis for cysteic acid peptides (Brown & Hartley, 1966). Soluble peptides from the various digests of rennin were applied as bands on Whatman 3MM paper and separated by high-voltage paper electrophoresis at pH 6.5. Strips cut from these preparative sheets were exposed to performic acid vapour and re-run at pH 6.5 to yield the diagonal 'maps' shown in Figs. 1-4, stained with cadmium-ninhydrin reagent (Heilmann, Barollier & Watzke, 1957).

Purification of the cysteic acid peptides. The major bands of cystine peptides thus located in the original preparative electrophoresis were cut out and exposed to performic acid vapour. Electrophoresis at pH 6.5 was then often sufficient to purify the cysteic acid peptides from 'diagonal' contaminants, but, when a cysteic acid peptide lay close to or within the diagonal band of other peptides, electrophoresis was first carried out at pH 2. Peptides containing two cysteic acid residues are anionic at this pH, and this is a useful test for such character. Peptides with one cysteic acid residue and no basic amino acid are neutral at pH 2, and can be subsequently resolved by electrophoresis of this neutral band at pH 6.5. All other peptides are cationic at pH 2 and move away from the cysteic acid peptides.

Amino acid analysis and sequence methods. These were as described by Smillie & Hartley (1966) and Brown, Kauffman & Hartley (1967). The 'dansyl'-Edman method (Gray & Hartley, 1963) was the main technique used for sequence determinations.*

Tryptic digestion of rennin. A typical tryptic digestion took place as follows. A 525 mg. portion of rennin (approx. 17 μ moles) was dissolved by dialysis against distilled water. The rennin solution, pH 5.8 (8 ml.), was transferred to a pH-stat and the rennin was denatured by rapid addition of 0.1 N-NaOH to pH 9. No autolysis appeared to occur, since there was no further alkali uptake. Trypsin (24 mg. dissolved in 2 ml. of water) was added to the rennin solution, and the digest was titrated at pH 9.0 at 20° by the addition of 0.1 N-NaOH. After 1 hr. the titration curve levelled out, and 30 min. later the digestion was stopped by addition of 0.1 N-HCl to pH 5.5. The alkali uptake during the tryptic digestion corresponded to approx. 12.3 moles/mole of rennin. Amino acid analyses (Foltmann, 1964) have indicated eight lysine and five arginine groups/mol. of rennin. During the digestion a slight turbidity developed, and at pH 5.5 the greater part of the digest precipitated as insoluble 'core peptides'.

Soluble peptides from the tryptic digest. The supernatant from the tryptic digest of denatured rennin was fractionated

by paper electrophoresis at pH 6.5. The only significant ninhydrin-positive bands were neutral or basic. Fig. 6 shows a peptide 'map' of the basic peptides, obtained by stitching a strip from the preparative pH 6.5 electrophoresis and subjecting this at right angles to descending chromatography in butan-1-ol-acetic acid-water (3:1:1, by vol.). The peptides A, B and C were purified by preparative chromatography of appropriate bands from the main sheets in butan-1-ol-acetic acid-water (3:1:1, by vol.) and eluted.

The neutral band of the original pH 6.5 electrophoresis was submitted to electrophoresis at pH 2. A peptide 'map' from a marker strip of this (Fig. 6) revealed three major neutral peptides, D, E and F, which were purified preparatively by chromatography as above.

Insoluble peptides from the tryptic digest. The peptides insoluble at pH 5.5 were dissolved in 50 ml. of 8 M-urea in 30 mM-tris-HCl buffer, pH 8.0, and applied to a column (50 cm. $\times$ 3 cm. diam.) of DEAE-cellulose equilibrated with the same 8 M-urea buffer. The peptides were eluted with a linear gradient of NaCl in the same buffer and detected both by E_{280} measurements and by a modification of the Folin-Lowry colour reaction for proteins (Lowry, Rosebrough, Farr & Randall, 1951) developed for the Technicon Auto-Analyser. Fractions were pooled as indicated in Fig. 8 and desalted either on columns of Sephadex G-25 or by precipitation at pH 5 after a short dialysis. The homogeneity of each fraction was tested by amino acid analysis and by digesting approx. 0.2 μ mole with chymotrypsin (18 hr. at 20° at pH 8.0, enzyme/substrate molar ratio 1:100). Peptide 'maps' were obtained by paper electrophoresis of these digests at pH 6.5 followed by chromatography in butan-1-ol-acetic acid-water (3:1:1, by vol.).

RESULTS

Diagonal 'maps' of cysteic acid peptides from rennin

The diagonal electrophoretic technique of Brown & Hartley (1963, 1966) provides a selective purification of peptides derived from the cystine bridges of a protein. Rennin contains six half-cystine residues/mol. of mol.wt. 30 700 (Foltmann, 1964), but we found considerable difficulty in finding conditions of digestion that revealed all of the disulphide bridges in a single diagonal 'map'. In most cases a considerable part of the protein remained as an insoluble 'core'. We therefore isolated soluble cysteic acid peptides from several different enzymic digests of rennin, but in all cases the peptides appear to derive from three unique cystine bridges, hereafter described as A, B and C.

Fig. 1 shows the diagonal 'map' of the soluble peptides from the rennin digest of denatured rennin. The only significant peptides are an acidic group (1A1, 1A2 and 1A3) that all derive from the acidic bridge A. Bridge C (the 'basic loop') is represented by a smear of peptides (1C), lying off the diagonal in the basic region of this 'map', that were not investigated further. No peptides could be detected in this digest that derive from the cystine bridge B,

* Abbreviations: DNS, 1-dimethylaminonaphthalene-5-sulphonyl; Cya, cysteic acid; Asx, asparagine or aspartic acid; Glx, glutamine or glutamic acid; AECys, *S*-amino-ethylcysteine.

Fig. 1. pH 6.5 diagonal 'map' of a rennin digest of denatured rennin. Conditions were as described in the text. Major cysteic acid peptides are hatched and numbered as in Table 1. Mobilities are expressed relative to that of 1-dimethylaminonaphthalene-5-sulphonic acid (-1.0) ($\bullet$).

Fig. 2. pH 6.5 diagonal 'map' of a peptic digest of the rennin 'core'. Details are as given in Fig. 1.

so it appears that rennin does not readily split bonds in this region of the chain.

The considerable amount of insoluble 'core' in the rennin digest did, however, dissolve after treatment with pepsin. Fig. 2 shows the diagonal 'map' of the peptic peptides derived from this 'core'. Bridge A is represented by peptides 2A1 and 2A2, which closely resemble peptides 1A1 and 1A2 in mobility. Evidently the prolonged rennin digestion has not completely split the bonds around bridge A. Two major peptides (2B1 and 2B2) are now seen that arise from bridge B. Peptide 2B1 clearly has no partner and is therefore likely to be derived from an internal cystine loop, and we show below that peptide 2B2 is also derived from this internally bridged sequence. The other cysteic acid peptides lying off the diagonal could arise from bridge C, but are present in insignificant amounts.

The most convenient digest for obtaining peptides from bridge C was the soluble chymotryptic fraction. Fig. 3 shows a major peptide (3C1), lying off this diagonal, that was cationic before oxidation and slightly anionic in the cysteic acid form. Its mobility, as shown below, is consistent with a content of two lysine residues and two cysteic acid residues, one of which is *N*-terminal. Since it has no partner the two half-cystine residues must have been internally bridged. Another significant band lying off the diagonal is peptide 3A1, which is

Fig. 3. pH 6.5 diagonal 'map' of a chymotryptic digest of rennin. Details are as given in Fig. 1.

similar to peptide 1A1. It appears to have been disulphide-bridged to a family of peptides arising from incomplete and non-specific chymotryptic cleavages in the other half of bridge A.

Fig. 4 is the only diagonal 'map' in which peptides from all three disulphide bridges are simultaneously

Fig. 4. pH 6.5 diagonal 'map' of a peptic digest of the chymotryptic 'core'. Details are as given in Fig. 1.

represented. It was obtained when the insoluble chymotryptic 'core' from the above treatment was further digested with pepsin. Bridge A is represented by the acidic peptides 4A1–4A5. Bridge B again appears as two internal loop peptides, 4B1 and 4B2, and bridge C also demonstrates the clear character of an internal loop in peptide 4C1. We are therefore able at this stage to describe the three disulphide bridges of rennin as an 'acidic bridge' (A), an 'acidic loop' (B) and a 'basic loop' (C).

Amino acid analyses of the cystine peptides

The amino acid analyses of the cystine peptides isolated from the digests described above are compiled in Table 1.

It appears from these analyses that most of the off-diagonal peptides in the acidic region of the diagonal 'maps' derive from the same cystine bridge. All these peptides are marked 'A' in the Figures.

Table 1. *Compositions of cysteine acid peptides from various digests of rennin*

Compositions are expressed as uncorrected ratios of amino acids after 16–20 hr. hydrolysis. Electrophoretic mobilities at pH 6.5 are expressed relative to that of 1-dimethylaminonaphthalene-5-sulphonic acid (–1.0). Colours of peptides after reaction with cadmium–ninhydrin reagent (Heilmann *et al.* 1957) on paper are: 'Slow R', red developing fully only after 12–18 hr. (indicative of *N*-terminal valine or isoleucine); 'Y→R', yellow becoming red after 12–18 hr. (indicative of *N*-terminal cysteic acid).

Peptide	Mobility at pH 6.5	Colour	Composition	Comment
1A1	–0.80	Y→R	Cya (0.5), Thr (0.8), Ser (0.9), Gly (0.9), Phe (1.2)	
1A2	–1.40	Red	Cya (0.9), Asp (4.0), Ser (0.4), Glu (0.6), Gly (0.8), Ile (1.0), Leu (1.5)	Contaminated with peptide 1A3?
1A3	–1.50	Red	Cya (0.9), Asp (3.7), Ser (1.0), Glu (1.0), Gly (1.0), Ile (1.1), Leu (1.2)	
1A3S1	–1.55	Red	Cya (1.2), Asp (3.8), Glu (0.8), Ile (1.0), Leu (0.9)	
1A3S2	–1.45	Red	Cya (0.9), Asp (3.6), Ile (1.0), Leu (1.1)	
1A3S3	–1.70	Red	Cya (1.0), Asp (3.0), Ile (1.0)	
2A1	–0.80	Red	Cya (0.8), Asp (1.1), Thr (1.5), Ser (2.0), Glu (2.6), Gly (2.1), Ala (0.8), Phe (1.2), Ile (0.7), Leu (0.6)	Impure?
2A2	–1.45	Slow R	Cya (0.9), Asp (2.8), Ile (1.0), Leu (1.1)	
2B1	–1.50	Red	Cya (1.9), Glu (2.0), Gly (1.8), Ala (1.0)	
2B2	–1.70	Y→R	Cya (1.6), Glu (2.0), Gly (2.1)	
3A1	–0.75	Orange	Cya (0.8), Asp (1.3), Thr (1.8), Ser (1.9), Glu (2.2), Gly (2.0), Phe (1.9)	<i>N</i> -Terminal threonine
3C1	–0.25	Y→R	Cya (1.8), Asp (2.0), Ser (1.1), Ala (1.1), Lys (1.9)	
4A1	–0.60	Red	Cya (1), Asp (1), Thr (2), Ser (4), Glu (2), Gly (3), Val (1.2), Phe (2)	Traces only: analyses approximate
4A2	–0.70	Red	Cya (1), Asp (1), Thr (1.2), Ser (2), Glu (3), Gly (3), Ala (3), Val (1.2), Ile (2), Leu (2), Phe (2)	
4A4	–1.30	Red	Cya (1), Asp (4), Thr (1.2), Ser (3), Glu (3), Gly (3), Ala (2), Val (1.2), Met (1), Ile (1), Leu (2), Phe (1)	
4A3	–0.75	Orange	Cya (0.7), Asp (1.0), Thr (1.7), Ser (2.1), Glu (2.1), Gly (2.1), Phe (1.7)	
4A5	–1.50	Red	Cya (0.8), Asp (3.8), Ile (1.1), Leu (1.1)	
4A5–3	–1.60	Y→R	Cya (1.0), Asp (1.9), Leu (1.1)	Three residues removed by Edman degradation
4B1	–1.45	Red	Cya (1.8), Glu (2.0), Gly (2.0), Ala (0.8)	
4B2	–1.65	Y→R	Cya (1.6), Glu (2.0), Gly (2.1)	
4C1	–0.20	Y→R	Cya (1.8), Asp (1.9), Ser (1.1), Ala (1.1), Lys (++)	Lysine by qualitative paper electrophoresis

Sequence of bridge A. The sequence of the acidic half of bridge A was established mainly by studies of peptide 1A3 (Cya, Asp₄, Glu, Gly, Ser, Ile, Leu). Paper electrophoresis at pH 2 of the products of a carboxypeptidase digest of peptide 1A3 showed that either serine, leucine or isoleucine was liberated. Since we later found leucine and isoleucine to be internal, serine must be C-terminal. A subtilisin digest of about 0.4 μ mole of this peptide was fractionated by

Fig. 5. Sequences around the disulphide bridges of rennin. Peptides are numbered as in Table 1, Figs. 1-4 and the text. The symbol — indicates the quantitative composition of the peptide; $\rightarrow$ indicates sequential analysis by the 'dansyl'-Edman method (Gray & Hartley, 1963); $\leftarrow$ indicates C-terminal sequences determined by relative liberation by carboxypeptidase.

electrophoresis at pH 6.5 to give free serine and glycine plus three peptides whose compositions are shown in Table 1 to be: peptide 1A3S1, (Cya, Asp₄, Glu, Ile, Leu); peptide 1A3S2, (Cya, Asp₄, Ile, Leu); peptide 1A3S3 (Cya, Asp₃, Ile). Peptide 1A3S1 is more anionic than peptide 1A2S2, so the extra glutamic acid residue in the former cannot be in the amide form. Peptide 1A3S2 has one aspartic acid residue and one leucine more than peptide 1A3S3, but since it is less anionic than the latter the extra aspartic acid is probably present as asparagine. Peptide 1A3S2 turned out to be identical with peptide 4A5, and peptide 2A2 merely lacked the *N*-terminal aspartic residue of the latter.

The results of 'dansyl'-Edman analysis of all these peptides are summarized in Fig. 5. Together with the observation that peptide 1A3S2 gave leucine with carboxypeptidase A they are sufficient to establish the sequence of this half of bridge A, but leave ambiguous the question of which aspartic acid residues are in the amide form. This question was resolved by examining the electrophoretic mobilities at pH 6.5 of each of the DNS-peptides derived from peptide PA2 after every round of Edman degradation (Table 2). Removal of a neutral residue should increase the electrophoretic mobility; removal of an acidic residue will decrease the anionic mobility. Only when isoleucine is removed does the mobility increase, so both of the *N*-terminal aspartic acid residues must be present in the acid form. We then isolated peptide 4A5-3, the product of three rounds

of Edman degradation of peptide 4A5. This had the expected composition (Cya, Asp₂, Leu) and an electrophoretic mobility consistent with two negative charges (Offord, 1966) (Table 1). Since the fourth aspartic acid residue has already been identified as asparagine, the third must be the free acid.

The sequence of the other half of bridge A was determined mainly by studies of the chymotryptic peptide 3A1 and the rennin peptide 1A1. Carboxypeptidase digestion of peptide 3A1 liberated phenylalanine together with small amounts of glycine and serine, and peptide 1A1 also showed *C*-terminal phenylalanine by the carboxypeptidase assay. Application of the 'dansyl'-Edman method gave the complete sequence of peptide 1A1 and the first six residues of peptide 3A1. In the latter case the electrophoretic mobilities at pH 6.5 of the degraded peptides were determined after each cycle of Edman degradation before reaction with DNS chloride. Table 2 demonstrates that the third residue must be glutamine rather than glutamic acid, the mobility decrease at the next step points to the removal of a negatively charged aspartyl residue, and the subsequent rise after removing the fifth residue points to glutamine rather than glutamic acid.

A slightly larger fragment of this sequence was isolated from one peptic digest. Peptide PA1 (Fig. 5) was essentially the same as peptide 3A1 but contained an additional *N*-terminal tyrosine residue. Its partner in the diagonal 'map' (peptide PA2) was identical with peptide 4A5. Papain digestion of peptide PA1 followed by electrophoresis at pH 3.5 yielded peptides PA1a-PA1e, whose compositions and *N*-terminal residues (Fig. 5) help to confirm the sequence determined above.

Sequence of bridge B. The peptides that arise from this bridge were obtained by peptic digestion of the insoluble 'core' from a rennin digest (peptides 2B1 and 2B2) or a chymotryptic digest (peptides 4B1 and 4B2). Table 1 shows that they have almost identical compositions: peptide 2B1 and 4B1, (Cya, Glu₂, Gly₂, Ala); or peptide 2B2 and 4B2, (Cya, Glu₂, Gly₂); and the diagonal 'maps' (Figs. 2 and 4) show that they must have arisen from an internal cystine bridge. The sequence shown in Fig. 5 was determined by 'dansyl'-Edman analysis of peptide 2B2, but leaves ambiguous the question whether the glutamic acid residues may have been present as glutamine. The electrophoretic mobility of the parent cystine peptides (Figs. 2 and 4) indicates one glutamic acid residue. This ambiguity was resolved, as before, by electrophoresis at pH 6.5 of the DNS-peptides at each stage of the Edman degradation of peptide 2B1. The electrophoretic mobilities shown in Table 2 indicate that the second residue removed must have been acidic, i.e. glutamic acid. The mobility of DNS-peptide 2B1-4 corresponds to a peptide with mol.wt. approx. 550 and

Table 2. *Electrophoretic mobilities of peptides produced by Edman degradation of peptides PA2, 2B1 and 3A1*

Electrophoretic mobilities at pH 6.5 are expressed relative to that of 1-dimethylaminonaphthalene-5-sulphonic acid (-1.0).

Peptide	Residue removed	Mobility at pH 6.5	
PA2	—	-1.50	DNS-peptide
PA2-1	Asp	-1.38	
PA2-2	Ile	-1.55	
PA2-3	Asp	-1.43	
PA2-4	Cya	-1.10	
2B1	—	-1.50	DNS-peptide
2B1-1	Cya	-1.38	
2B1-2	Glu	-1.10	
2B1-3	Gly	-1.17	
2B1-4	Gly	-1.36	
3A1	—	-0.75	Unmodified peptide
3A1-1	Thr	-0.78	
3A1-2	Ser	-0.82	
3A1-3	Gln	-0.87	
3A1-4	Asp	-0.48	
3A1-5	Gln	-0.52	

two negative charges, i.e. DNS-Cya-Gln rather than DNS-Cya-Glu (Offord, 1966).

Sequence of bridge C. Peptides 3C1 and 4C1 (Table 1) have identical compositions (Cya₂, Asp₂, Ser, Ala, Lys₂) and their amino acid sequence was determined almost entirely by the 'dansyl'-Edman

method (Fig. 5). The sequence evidence was completed by examination of two other peptides isolated from a combined tryptic plus chymotryptic digest of denatured rennin. Peptide CT1 had the composition: Cya (1.0), Lys (1.0); peptide CT2 had the composition: Cya (0.9), Asp (1.0), Ser (0.9), Ala (1.0), Lys (1.1). Both peptides were neutral at pH 6.5, so peptide CT2 must contain asparagine. The *N*-termini of these peptides confirm the sequence for bridge C shown in Fig. 5.

The electrophoretic behaviour of peptide 3C1 in the diagonal 'map' (Fig. 3) confirms the presence of two net positive charges before oxidation, so no free aspartic acid could be present. The slight anionic mobility at pH 6.5 after oxidation is probably due to an abnormally low *pK* for the α -amino group of the cysteine acid residue.

Sequence of soluble peptides from a tryptic digest of rennin

Fig. 6 shows the 'fingerprint' of the soluble tryptic peptides from denatured rennin. The purification of these peptides is described above and their amino acid compositions are shown in Table 3.

Fig. 7 summarizes the evidence for the sequence of these peptides. The 'dansyl'-Edman method was sufficient to determine peptides B, D, E and F, but for peptide C further digestion with chymotrypsin was used (18 hr. at 20° at pH 8.0, enzyme/substrate molar ratio 1:100). Electrophoresis at pH 2 gave peptides CC1, CC2, CC3 and CC4, whose sequences are shown in Fig. 7. The electrophoretic

Fig. 6. Peptide 'maps' of soluble peptides from a tryptic digest of rennin. (a) Basic peptides obtained by stitching a strip of cationic peptides separated at pH 6.5 for descending chromatography in butan-1-ol-acetic acid-water (3:1:1, by vol.). (b) Neutral peptides obtained by re-running the neutral band after pH 6.5 at pH 2; a strip from this was then chromatographed as above.

Table 3. *Compositions of peptides from rennin*

Electrophoretic mobilities at pH 2 are expressed relative to that of glycine (+1.0). (+) indicates basic residues determined by qualitative paper electrophoresis, or tryptophan by Ehrlich's reaction of the peptide on paper. Other details are as given in Table 1.

Peptide	Mobility		Colour	Composition
	pH 6.5	pH 2		
<i>Peptides from tryptic digest of denatured rennin</i>				
A	+ 1.20	—	Red	Free Lys
B	+ 0.70	—	Yellow	His (1.0), Arg (1.0), Asp (1.1), Glu (1.3)
C	+ 0.35	—	Red	Lys (1.0), Asp (1.8), Gly (1.1), Ala (2.2), Val (1.0), Leu (1.9)
D	0	0.9	Red	Lys (0.8), Asp (1.1), Glu (1.0), Gly (1.1), Ile (1.0)
E	0	0.9	Red	Arg (0.9), Asp (1.1), Pro (1.1), Phe (0.9)
F	0	0.7	Red	Ala (1.0), Ile (1.0)
G	Insoluble	—	—	Trp (+), Arg (1.0), Asp (1.2), Gly (1.1), Val (0.9), Ile (1.8), Leu (1.0), Phe (0.9)
<i>Peptides from other digests of rennin</i>				
R1	0	1.0	Red	Arg (1.0), Glu (1.8), Ile (1.1)
R2	0	0.7	Red	Arg (+), Asp (2.8), Ala (0.9), Leu (1.0)
RC1	+ 0.70	—	Red	Lys (+), Ala (2.0), Ile (1.0)
RC2	0	0.7	Red	Arg (+), Asp (2.0), Ala (1.0), Leu (0.9)
TC1	+ 0.60	—	Red	Arg (+), Asp (1.0), Met (0.9)
TC2	+ 0.30	—	Red	Lys (0.8), His (0.8), Asp (1.1), Ser (2.0), Glu (3.1)
TC3	— 0.90	—	Red	Asp (1.1), Thr (1.0), Ser (1.0), Glu (2.6), Pro (0.8), Gly (1.3), Val (1.1), Phe (1.0)

Peptides from the tryptic digest		Peptides from other digests	
A: Lys		R1: Ile-Gln-Arg-Glu	
B: Asn-His-Gln-Arg		R1T1	R1T2
C: Ala-Asn-Asn-Leu-Val-Gly-Leu-Ala-Lys		R2: Asp-Arg-Ala-Asn-Asn-Leu	
CC1	CC2	R2T1	R2T2
CC3	CC4	RC1: Ala-Lys-Ala-Ile	
D: Glx-Ile-Asx-Gly-Lys		RC2: Asx-Ala-Asx-(Arg,Leu)	
E: Phe-Asp-Pro-Arg		TC1: Met-Asn-Arg	
F: Ala-Ile		TC2: Glx-Ser-Glx-Asx-His-Ser-Gln-Lys	
G: Trp-Ile-Leu-Gly-Asp-Val-Phe-Ile-Arg		TC2S3	TC2S2
GC1	GC2	TC2S1	
		TC3: Ser-Thr-Gln-Glu-Pro-Gly-Asx-Glx-Val-Phe	
		TC3N1	TC3N2
			TC3N3

Fig. 7. Sequences of peptides from various digests of rennin. Peptides A-F were soluble tryptic peptides. Peptide G was from peak II of Fig. 8. Peptides R1 and R2 were from rennin autolysates, and peptides RC1 and RC2 from chymotryptic digests of the rennin 'core'. Peptides TC1, TC2 and TC3 were chymotryptic fragments of insoluble tryptic peptides (Fig. 8). Symbols are as given in Fig. 5, but — indicates *N*-terminal tryptophan determined as the carbamoyl derivative (see the text).

mobility of these peptides indicates that peptide B contains both asparagine and glutamine, peptide C has two asparagine residues, peptide E has aspartic acid, and peptide D must contain one acidic residue that could be either aspartic acid or glutamic acid. Only 25 of the 270 residues in rennin are found in these soluble tryptic peptides, and only two of the five arginine residues and three of the eight lysine residues are accounted for. Hence over 90% of the residues must occur in eight tryptic peptides that are insoluble at pH 5.5. Peptide F probably arises from the *C*-terminus of rennin, which has been reported to be leucine or isoleucine (Jirgensons, Ikenaka & Gorguraki, 1958).

Insoluble peptides from the tryptic digest

The insoluble portion of the tryptic digest was fractionated in 8M-urea solutions on a column of DEAE-cellulose as described in the Methods section. Fig. 8 shows the elution profile of this column. Although the separation looked promising, amino acid analysis and chymotryptic peptide 'maps' of fractions from the peaks of Fig. 8 showed that all but one of the peaks were cross-contaminated. Further attempts to purify each fraction on

Sephadex columns were relatively unsuccessful. The exception was peak II, which contained a single peptide, G, whose composition and sequence are shown in Table 3 and Fig. 7. Actually, when isolated, peptide G was obtained in a partially *N*-carbamoylated form, which probably arose when urea solutions of the peptide were stored at alkaline pH. Chymotryptic digestion of peptide G (18 hr. at room temperature at pH 8.5) gave the basic peptide GC3 (mobility +0.85 at pH 6.5), the acidic peptide GC2 (mobility -0.54 at pH 6.5) and free tryptophan in the neutral band. However, an additional acidic ninhydrin-negative Ehrlich-positive band (GC1) was observed that appeared about one-third as intense as the free tryptophan. No amino acids were found after acid hydrolysis of this, but it had the same mobility at pH 6.5 (-0.9) and pH 2.0 (0.0) as synthetic *N*-carbamoyl-L-tryptophan prepared by the method of Stark, Stein & Moore (1960). Hence peptide G must have *N*-terminal tryptophan and its sequence was determined as shown in Fig. 7.

Miscellaneous peptides from other digests

Table 3 and Fig. 7 give the compositions and sequence of a few other peptides from various

Fig. 8. Chromatography of insoluble tryptic peptides in 8M-urea in 30mM-tris-HCl buffer, pH 8.0, on a column (50cm. $\times$ 3cm. diam.) of DEAE-cellulose. —, E_{280} ; - - - - - , Folin-Lowry colour (Lowry *et al.* 1951); — · — · — , concn. of NaCl in effluent.

digests of denatured rennin that help to extend our knowledge of its sequence.

Peptides R1 and R2 were soluble neutral peptides in a rennin autolysate, which were further purified by electrophoresis at pH 2 and chromatography in butan-1-ol-acetic acid-water. Trypsin attacked peptide R1 very slowly to give peptide R1T1 (mobility +0.70 at pH 6.5) and free glutamic acid. Tryptic digestion of peptide R2 gave two neutral peptides, R2T1 and R2T2, which were purified by electrophoresis at pH 2 (mobilities +1.10 and +0.50 respectively at pH 2). Carboxypeptidase gave leucine plus traces of asparagine with peptide R2T2.

The basic peptides from a pH 6.5 electrophoresis of this rennin autolysate were eluted and digested with chymotrypsin (18 hr. at 20° at pH 8.0, enzyme/substrate molar ratio 1:100). Peptide RC1 was purified by electrophoresis at pH 6.5 and pH 3.5, and peptide RC2 was neutral at pH 6.5 and was purified by electrophoresis at pH 2.

The composition and sequence of three other peptides are shown in Table 3 and Fig. 7, these being derived from chymotryptic digests of the tryptic fractions from the DEAE-cellulose column shown in Fig. 8. Peptide TC1 was obtained in good yield from fractions VIII and IX (Fig. 8) and as a minor component of fractions X and XI. Peptide TC2 was the major basic peptide in fractions X and XI and was in lower yield in fraction VIII. Also in high yield in fraction X was peptide TC3, which may therefore be part of the tryptic peptide whose C-terminal sequence is that of peptide TC2.

The sequence of these three chymotryptic fragments of the tryptic 'core' is shown in Fig. 7. Subtilisin digestion of peptide TC2 gave two basic

peptides, TC2S1 and TC2S2, and a neutral peptide, TC2S3; hence one free acidic group must be present in the first four residues of this peptide. Peptide TC3 gave phenylalanine plus traces of valine with carboxypeptidase. Digestion with papain gave mainly peptide TC3N1 (neutral at pH 6.5) and peptide TC3N2 (mobility -1.10 at pH 6.5), and a minor component was peptide TC3N3 (mobility -0.83 at pH 6.5). These mobilities suggest that one of the last two acidic residues carries an amide group.

DISCUSSION

We found that most enzymic digests of denatured rennin gave considerable amounts of insoluble 'core' peptides that could not be fractionated by paper electrophoresis. Nevertheless, the selective purification procedure for cysteic acid peptides that is implicit in the diagonal electrophoretic technique enabled us to select soluble cysteic acid peptides from a number of different digests. In all cases these appeared to arise from the three unique stretches of sequence shown in Fig. 5.

It is remarkable that, in a chain of 270 residues, two of the three disulphide bridges should be in the form of small loops containing five or six amino acid residues. Further, this feature is also found in the disulphide bridges of hog pepsin (J. Tang & B. S. Hartley, unpublished work), as shown in Fig. 9. The sequence of bridge C is extended in Fig. 9 by postulating an overlap between peptide 4C1 (Fig. 5) and the tryptic peptide B (Fig. 7). Tryptic digestion of the former sequence must give rise to a tryptic peptide with *N*-terminal asparagine and peptide B is the only such peptide that meets this criterion.

Only about 10% of the sequence of rennin (25 residues) appears in the six soluble tryptic peptides shown in Fig. 7. The amino acid analysis of rennin (Foltmann 1964) indicates eight lysine residues and five arginine residues so we would expect to find the remaining 245 residues in three arginine peptides and five lysine peptides in the tryptic core. One of these arginine peptides (G; Fig. 7) was purified and another peptide with C-terminal -Met-Asn-Arg (peptide TC1; Fig. 7) is almost certainly the major arginine peptide in fraction VIII (Fig. 8). Similarly peptides TC2 and TC3 probably come from the major lysine peptide in fraction X. We might expect to find the missing arginine sequence or the four missing lysine sequences in peptides from other digests of denatured rennin.

We actually find three other arginine sequences in autolysates of rennin. Fig. 7 shows that peptides R1, R2 and RC2 all differ in sequence from the other arginine peptides (B, E, G and TC1). This could mean that rennin A and rennin B, which are both present in the crystalline rennin that we used in this study, differ in sequence around their arginine

Bridge A:

Bridge B:

Bridge C:

C-Terminus:

Fig. 9. Homologies between calf rennin and hog pepsin. Identical residues are in boxes and chemically similar residues are in **bold** type. For pepsin, disulphide-bridge sequences are those determined by J. Tang & B. S. Hartley (unpublished work) and C-terminal sequences are from Dopheide *et al.* (1967).

residues. However, we prefer another explanation. Prorennin contains seven arginine residues (Foltmann 1964). The peptides that are liberated from it in the process of activation tend to bind to the rennin that is formed during passage through a Sephadex G-25 column (B. Foltmann, unpublished work). It is therefore possible that the two extra arginine peptides that we find in autolysates of rennin arise from bound activation peptides in the crystalline material. Peptides R1 and RC2 are present in relatively lower yield in such autolysates, and we suspect that these two sequences may be from prorennin especially since peptide R2 belongs to the C-terminus of rennin as shown below.

The tryptic peptide F (Fig. 7) is almost certainly the C-terminus since it is present in high yield in the tryptic digest, and Jirgensons *et al.* (1958) found that leucine or isoleucine is C-terminal in the chain.

Overlaps between the tryptic peptides C and F are found in the rennin peptides R2 and RC1. Hence we may write the C-terminal sequence of rennin as shown in Fig. 9. This is remarkably homologous with the C-terminal sequence of hog pepsin described by Dopheide *et al.* (1967), Perham & Jones (1967), Koehn, Oestreicher & Perlmann (1967) and Kuznetsov, Kovaleva & Stepanov (1966). Further, peptide G (Fig. 7) of rennin is remarkably homologous with part of this sequence and almost certainly must occupy an analogous position close to the C-terminus.

The degree of homology between calf rennin and hog pepsin is shown in Fig. 9. The sequences determined by J. Tang & B. S. Hartley (unpublished work) for bridge B and bridge C of hog pepsin are partially confirmed by two peptides, Ser-Ser-Leu-Ala-AECys and Ser-Gly-Gly-AECys, isolated by Dopheide *et al.* (1967), but they differ from those

reported by B. Keil, L. Morávek & F. Šorm (personal communication) for the same protein. If the degree of possible homology is maximized, as in Fig. 9, we find only four identities out of 15 comparable residues in bridge A, whereas bridge B (four identities in six residues) and bridge C (seven identities in 11 residues) are much more homologous. The C-termini of calf rennin and hog pepsin are very similar: 17 out of 21 comparable residues are identical, and this stretch of the molecule is notable, as pointed out by Dopheide *et al.* (1967), in containing the only two arginine residues and single lysine residue of hog pepsin. The total homology so far determined is 28 out of 38 comparable residues (74% identity), a value which exceeds that indicated at a somewhat similar preliminary stage for the pancreatic proteinases bovine chymotrypsin and porcine elastase (52%) (Brown *et al.* 1967).

Note added in proof. We have recently isolated a peptide from tryptic digests of rennin whose sequence is Glu-Tyr-Tyr-Ser-Val-Phe-Asp-Arg. This is identical with a sequence in pepsin (Fig. 9) except that serine replaces threonine, glutamic acid replaces glutamine and the homology between rennin and pepsin is extended to 21 out of 27 residues at the C-terminus.

B. F. is grateful to the Royal Danish School of Pharmacy for a grant as research associate and to the Carlsberg Foundation and the European Molecular Biology Organisation for travelling expenses.

REFERENCES

- Bang-Jensen, V., Foltmann, B. & Rombauts, W. (1964). *C.R. Lab. Carlsberg, Sér. chim.* **34**, 326.
 Brown, J. R. & Hartley, B. S. (1963). *Biochem. J.* **89**, 59P.
 Brown, J. R. & Hartley, B. S. (1966). *Biochem. J.* **101**, 214.
 Brown, J. R., Kauffman, D. L. & Hartley, B. S. (1967). *Biochem. J.* **103**, 497.
 Dopheide, T. A. A., Moore, S. & Stein, W. H. (1967). *J. biol. Chem.* **242**, 1833.
 Foltmann, B. (1959). *Acta chem. scand.* **13**, 1927.
 Foltmann, B. (1962). *C.R. Lab. Carlsberg, Sér. chim.* **32**, 425.
 Foltmann, B. (1964). *C.R. Lab. Carlsberg, Sér. chim.* **34**, 275.
 Foltmann, B. (1966). *C.R. Lab. Carlsberg, Sér. chim.* **35**, 143.
 Gray, W. R. & Hartley, B. S. (1963). *Biochem. J.* **89**, 379.
 Hartley, B. S., Brown, J. R., Kauffman, D. L. & Smillie, L. B. (1965). *Nature, Lond.*, **207**, 1157.
 Heilmann, J., Barollier, J. & Watzke, E. (1957). *Hoppe-Seyl. Z.* **309**, 219.
 Jirgensons, B., Ikenaka, T. & Gorguraki, V. (1958). *Makromol. Chem.* **28**, 96.
 Koehn, P. V., Oestreicher, A. B. & Perlmann, G. E. (1967). *J. biol. Chem.* (in the Press).
 Kuznetsov, Y. S., Kovaleva, G. C. & Stepanov, V. M. (1966). *Biochim. biophys. Acta*, **118**, 219.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Offord, R. E. (1966). *Nature, Lond.*, **211**, 591.
 Perham, R. N. & Jones, G. M. T. (1967). *Europ. J. Biochem.* (in the Press).
 Smillie, L. B. & Hartley, B. S. (1966). *Biochem. J.* **101**, 232.
 Stark, G. R., Stein, W. H. & Moore, S. (1960). *J. biol. Chem.* **235**, 3177.