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Carrageenans and the proteolytic activity of human gastric secretion

W. ANDERSON AND A. J. BAILLIE

The inhibition of the peptic activity of human gastric secretion by undegraded and degraded carrageenans of similar sulphate content has been examined over the pH range 1·5–3·75. Inhibition by degraded carrageenan is constant throughout this range, but inhibition by undegraded carrageenan decreases between pH 2·5 and 3·25, when a lower level is established. The inhibition by both types of carrageenan is caused by substrate-inhibitor interaction. The differences in degree of inhibition and the effect of pH on the inhibition by undegraded carrageenan appear to originate in the differing natures of the substrate-inhibitor complexes formed by degraded and undegraded carrageenans.

CARRAGEENANS inhibit the peptic activity of gastric juice but quantitative differences in antipeptic activity are to be found amongst carrageenans from different seaweeds and even from the same species of seaweed harvested in different seas. There are also differences in activity between the κ- and λ-components of the same carrageenans and between undegraded and degraded carrageenans of similar bound sulphate content from the same source (Anderson & Harthill, 1967).

For one degraded carrageenan it has been shown (Anderson, 1961) that interaction with substrate with consequent substrate occlusion, or depletion, is responsible for the observed antipeptic activity. Hence the factors affecting such interaction would be expected to influence antipeptic activity.

It was therefore of interest to study, over a pH range, carrageenans of different molecular weight and of similar bound sulphate content, to determine whether there is a common type and mechanism of inhibition of proteolysis for carrageenans, and to show whether known aspects of structure which would influence protein interaction do in fact give rise to differences in antipeptic activity. The effect of pH has added interest because it is now known that gastric juice contains several proteases with different pH optima for proteolysis (Tang, Wolf & others, 1959; Taylor, 1962; Seijffers, Miller & Segal, 1964).

We here report on the nature of the antipeptic activity displayed over a pH range by undegraded and degraded carrageenans.

Experimental

MATERIALS AND METHODS

Carrageenans. Undegraded carrageenan was the λ-carrageenan from *Chondrus crispus* (CY-λ) containing 37·3% bound sulphate (SO_3Na); the degraded carrageenan was a degraded κ-like carrageenan, derived from the κ-like carrageenan of *Eucheuma spinosum* by mineral acid degradation

From the Department of Pharmacy, University of Strathclyde, Glasgow.

under mild conditions and containing 36·1% SO_3Na. These carrageenans have been described and their antipeptic activity compared under standardized but necessarily restricted conditions (Black, Blakemore & others, 1965; Anderson & Harthill, 1967). Undegraded λ-carrageenan was chosen as an example of undegraded carrageenans because of the greater activity of λ-carrageenans compared with κ-carrageenans. Degraded *Eucheuma* (κ-like) carrageenan was chosen as an example of a degraded carrageenan because of the relative ease of extensive degradation of this type of carrageenan under mild conditions, and its consequent stability compared with degraded λ-carrageenans. The κ-λ differentiation in this respect is less important than the state of degradation.

Solutions of undegraded carrageenan were made by hydrating the substance by shaking in the appropriate buffer for up to 18 hr before adjusting volume; degraded carrageenan dissolved immediately.

Gastric secretion. Secretions obtained during augmented histamine tests were pooled and used after centrifugation to remove insoluble matter. Acid-free, blood- and bile-stained secretions were not used. They were obtained from ulcer patients through the courtesy of Dr. A. I. M. Glen, Western Infirmary, Glasgow.

ANTIPEPTIC ACTIVITY

(*a*) *Carrageenan added to enzyme* (*gastric juice*) *before digestion.* Digestion was by a method similar to that of Hunt (1948) at pH values between 1·5 and 3·75 using appropriate glycine buffers (Long, 1961). The substrate solution was 5% w/v whole human plasma protein dissolved in buffer at the appropriate pH. Any necessary adjustment of pH was by addition of dilute hydrochloric acid. The centrifuged gastric juice was diluted with an equal volume of buffer (control) or buffer containing the inhibitor (carrageenan). All solutions were at 37°. 1 ml of diluted gastric secretion or solution of carrageenan in gastric juice was added to 5 ml substrate solution and digestion allowed to proceed at 37° for 15 min, at the end of which trichloroacetic acid (10 ml, 0·35 N) was added. After 4 min the digests were filtered (Whatman No. 1) and to 2 ml filtrate, sodium hydroxide (20 ml, 0·25 N) was added, followed by 1 ml of Folin-Ciocalteu reagent, mixing being effected by swirling. Colour development required 15 min standing and the extinction was then read at 680 mμ. Following Hunt (1948), extinction values were converted to units. The differences between inhibited and uninhibited values was expressed as a fraction of the uninhibited after due allowance for blanks, which in addition to those prescribed by the method included one containing only gastric secretion and buffer. This fractional inhibition, i, which equals $1 - V_i/V$ where V_i and V represent digestion with and without inhibitor respectively, is also referred to as "inhibition".

(*b*) *Carrageenan added to substrate before digestion.* Amounts and concentrations of the components of the digestion mixture were as in (*a*) above. The appropriate amount of carrageenan in buffer solution was

added to a solution of substrate and mixed, followed by adjustment of volume and any necessary adjustment of pH. The diluted gastric secretion was then added and digestion was as in (*a*).

In methods (*a*) and (*b*), the digestion mixture (6 ml total) contained 2 mg of the undegraded carrageenan and 30 mg of the degraded carrageenan in solution.

(*c*) *Variation of substrate and inhibitor concentrations. Method* (*a*) was used at pH 2·2 and 3·2.

(i) With carrageenan concentration (I), constant at 1 mg undegraded, and 10 mg degraded, in the 6 ml digest, substrate concentrations (S) ranged from 100–300 mg. The same experiment was also performed in the absence of carrageenan.

(ii) Using two substrate concentrations (mg/6 ml digest), 125 and 250, the following carrageenan concentrations were used (mg/6 ml digest): 0, 0·15, 0·3, 0·6, 0·75, 1·25, 1·5, 2·0 (undegraded); and 0, 2·5, 10, 15, 20, 25, 30 (degraded).

In the digestion systems used, digestion increased linearly with time and substrate inhibition was absent.

SUBSTRATE-INHIBITOR INTERACTION

Increasing amounts of carrageenan in 0·2% sodium chloride solution were added to 1 ml volumes of plasma protein solution (0·25%) and the final volumes adjusted to 5 ml. The appearance of free carrageenan in the supernatant (detected by toluidine blue) indicated saturation of the protein. One series of experiments was made at pH 2·2, another at pH 3·2. The ratio mg protein : mg carrageenan at which free carrageenan first appeared in the supernatant is defined as the "saturation value" and is a measure of the protein-carrageenan interaction.

Results and discussion

Fig. 1 shows that when carrageenan was added to enzyme first, the inhibitions caused by the two carrageenans were similar up to pH 2·5, after which the inhibition caused by the undegraded carrageenan fell to a new level which was reached at pH 3·25, whilst the inhibition caused by degraded carrageenan remained reasonably constant throughout the pH range. It also shows that when the undegraded carrageenan was added to enzyme first, method (*a*), a significantly greater inhibition ($P < 0{\cdot}001$ at the pH of minimum difference in inhibition) always occurred than was obtained when substrate was added first. For degraded carrageenan no such difference occurred.

The marked difference in inhibition pattern between undegraded and degraded carrageenans as inhibitor concentration is increased at a constant pH of 2·0 in both methods (*a*) and (*b*), is shown in Fig. 2a, b; possible explanations of this difference in behaviour will be discussed later.

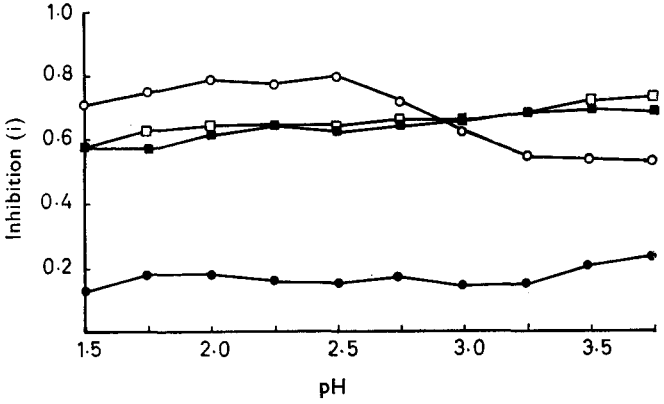

FIG. 1. Inhibition by two carrageenans of the peptic activity of human gastric secretion at various pH values; 6 ml digests contained: 0·5 ml gastric secretion, 250 mg plasma protein substrate, and either 2 mg undegraded, or 30 mg degraded carrageenan. ○, Undegraded carrageenan added to enzyme (gastric secretion) before addition of substrate. Significance of difference of inhibition by undegraded carrageenan at pH 2 and at pH 3·25, $P < 0{\cdot}01$. ●, Undegraded carrageenan added to substrate first. □, Degraded carrageenan added to enzyme first. ■, Degraded carrageenan added to substrate first. For undegraded carrageenan added to enzyme first each point is the mean of 20 determinations (n); added to substrate first, $n = 6$. For degraded carrageenan $n = 4$ in both instances.

Double reciprocal plots (Fig. 3a, b) and plots (Fig. 4a, b) of $1/V_i$ against I (Dixon, 1953) for undegraded and degraded carrageenans at pH 2·2 and pH 3·2 indicate substrate-inhibitor interaction as the mechanism of inhibition. Using the data of Figs 4 and 5, plots of S/V_i against S, V_i against V_i/S, and 1/i against 1/I also yielded curves typical (Webb, 1963) of substrate-inhibitor interaction.

Three mechanisms underlying inhibition of this general type can be distinguished (Reiner, 1959) by plotting I against i/(1−i). (*i*) Substrate depletion. (*ii*) Inhibition of the enzyme by a substrate-inhibitor complex. (*iii*) A combination of both mechanisms. The shape of such a plot for both undegraded and degraded carrageenans at pH 2·2 and pH 3·2 (Fig. 5a, b) is typical of inhibition effected by substrate depletion.

SUBSTRATE-INHIBITOR INTERACTION

In the substrate-inhibitor interaction studies, saturation values for undegraded and degraded carrageenans were approximately 4 for both types, indicating that the plasma substrate can remove one-quarter of its weight of either degraded or undegraded carrageenan, before free carrageenan appears in solution. In digestion studies maximum inhibition by degraded carrageenan is seen when the plasma protein-carrageenan ratio is also around 4, using methods (*a*) and (*b*). It therefore appears that maximum inhibition by degraded carrageenan is seen when the substrate is "saturated". In the case of undegraded carrageenan, however, although the saturation value is also about 4, maximum

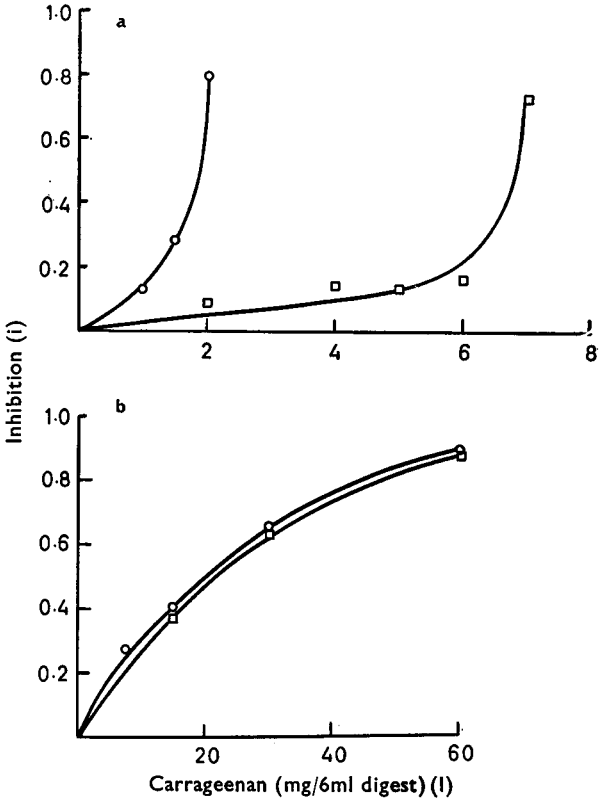

FIG. 2. Effect of increasing concentration of (a) undegraded, and (b) degraded carrageenan (I) on the inhibition (i) of the peptic activity of human gastric secretion. ○, Carrageenan added to enzyme first. □, Carrageenan added to substrate first.

inhibition is seen in method (*a*) when the substrate-inhibitor ratio by weight is about 125; in method (*b*) where the undegraded carrageenan displays lower activity, the ratio is reduced to 35 (Fig. 2a).

From Fig. 2a, b it can be calculated that, for i = 0·2 to 0·8 the ratio of weights of carrageenans degraded/undegraded varied between 5 and 23, method (*a*), and between 1 and 7, method (*b*).

THE DIFFERENCE BETWEEN UNDEGRADED AND DEGRADED CARRAGEENANS

Although undegraded and degraded carrageenans inhibit peptic activity by the same mechanisms, Figs 1 and 2 reveal points of difference. Since inhibition by carrageenan reflects interaction with protein substrate it would be remarkable if two carrageenans with such markedly different molecular weights (even though sulphate contents are similar) interacted with substrate protein similarly, to give identical inhibitions.

The results of the substrate-inhibitor interaction studies indicated that for undegraded carrageenan, saturation values were the same at pH 2·5

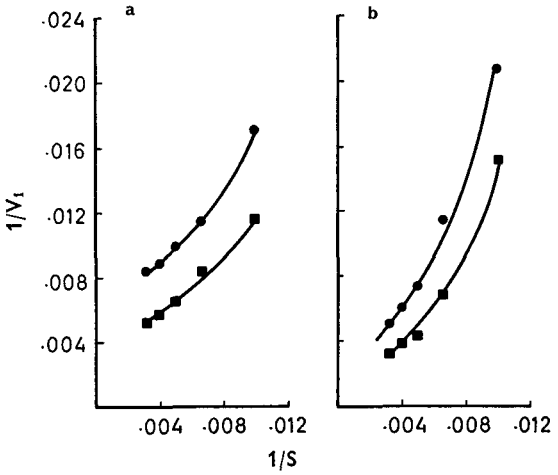

FIG. 3. Plot of $1/V_1$ against $1/S$ for (a) undegraded, and (b) degraded carrageenan at two pH values. 6 ml digest contained either 1 mg undegraded or 10 mg degraded carrageenan. ■, pH 2·2; ●, pH 3·2.

and pH 3·25, suggesting that the decrease in inhibition with pH (Fig. 1) was not due to quantitative difference in substrate-inhibitor interaction at the two pH levels. This suggests the possibility of qualitative differences at the two pH values in the interaction between undegraded carrageenan and protein responsible for the depletion or occlusion of substrate, one being more efficient in preventing access of enzyme than the other. Even allowing that at pH 3·25, peak gastricsin activity will occur, the possibility that, as an explanation of the results in Fig. 1, undegraded carrageenan acts principally on the proteolytic activity seen at pH 2·2 (pepsin) and not on that seen at pH 3·25 (gastricsin) is discounted by the similarity of the results in Figs 3–5 which do not point to a pH-dependent qualitative difference in inhibition type. With degraded carrageenan inhibition is unaffected by pH and there is therefore no reason to suspect that the structure of the protein-polysaccharide complex varies with pH in this range, at least insofar as it affects access of enzyme to protein.

With undegraded carrageenan the order of mixing of enzyme-substrate-inhibitor affects the level of inhibition and the appearances of the digests. Thus, when undegraded carrageenan is mixed with gastric secretion first, subsequent addition to substrate is followed by formation of large curds; the greater the amount of undegraded carrageenan the more colourless the supernatant becomes, suggesting greater depletion of protein from solution. On the other hand, when undegraded carrageenan is added to the substrate first, the appearance of the mixture after addition of gastric juice is similar to that seen when degraded carrageenan is used, a coarse precipitate of uniformly sized floccules being formed which are quite distinct in appearance from the curds. With the degraded carrageenan, order of addition affects neither the appearance of the digest nor the level of inhibition (Fig. 1).

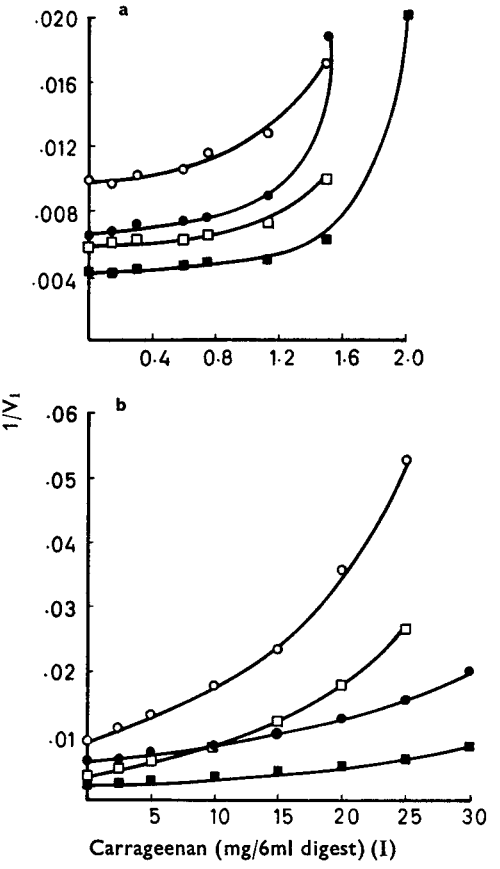

FIG. 4. Plot of $1/V_1$ against I for (a) undegraded, and (b) degraded carrageenan at two pH values and two substrate concentrations (S) (mg/6 ml digest). ○ = S,125; pH 3·2. ● = S,250; pH 3·2. □ = S,125; pH 2·2. ■ = S,250; pH 2·2.

Both types of carrageenan act by substrate depletion (Figs 3–5), but Fig. 2a, b reveal differences between the two carrageenans in rate of approach to complete inhibition with increasing inhibitor concentration. To examine this point a control experiment was made using decreasing amounts of substrate without inhibitor (to correspond to substrate depletion caused by inhibitor). A curve similar to Fig. 2a (undegraded carrageenan) was obtained when fractional reduction in digestion was plotted against decreasing substrate concentration (Fig. 6). Fractional reduction in digestion, r, equals $1 - v/V$ where V is the digestion at maximum substrate concentration and v is the digestion at various lower substrate concentrations.

The difference between Fig. 2a (undegraded carrageenan) and Fig. 2b (degraded carrageenan) suggests that substrate depletion (or occlusion) by degraded carrageenan from the enzyme system is not complete.

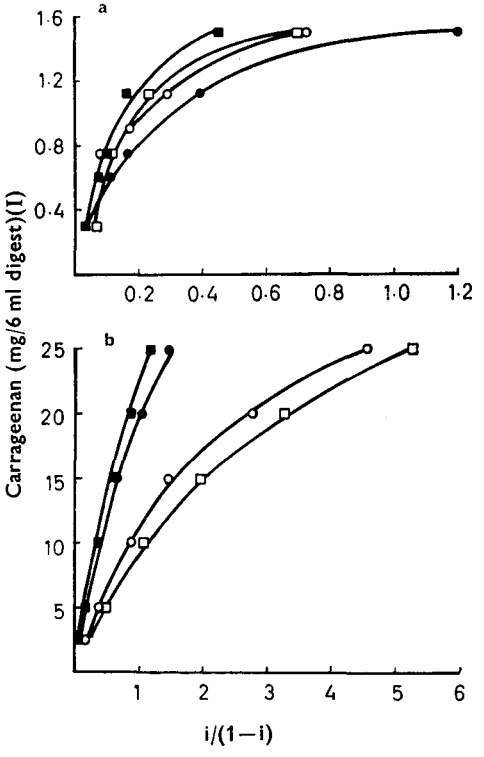

FIG. 5. Plot of I against i/1-i for (a) undegraded, and (b) degraded carrageenan at two pH values and two substrate concentrations (S)(mg/6 ml digest). ○ = S,125; pH 3·2 ● = S,250; pH 3·2. □ = S,125; pH 2·2. ■ = S,250; pH 2·2.

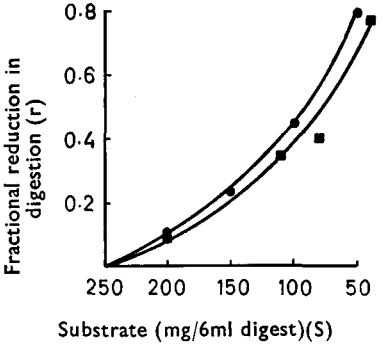

FIG. 6. The effect of decreasing substrate concentration, S, on fractional reduction (r) in digestion. ■ = pH 2·2; ● = pH 3·2.

These differences in inhibitory pattern between the two types of carrageenan could be explained by the differing molecular sizes. The larger carrageenan molecule with a large number of charged sites on the chains, more effectively binds greater amounts of protein substrate with the formation of a compact polysaccharide-protein barrier separating enzyme and substrate. The smaller, degraded carrageenan molecules on the other hand will interact with small numbers of protein molecules tending to form smaller complexes covered with a polysaccharide envelope which is less compact than that formed by the undegraded molecule, leaving some access for the pepsin to the protein unless high concentrations are used. This suggestion is in accord with the differences in appearance between the different protein-polysaccharide precipitates in the digests to which attention has already been drawn, and also with the additional amount of degraded carrageenan, compared with undegraded carrageenan, which is necessary for protection ($i = 0{\cdot}8$) of the substrate. In terms of molarities (molecular weight of undegraded carrageen = 800,000; degraded carrageenan = 25,000), the difference (at $i = 0{\cdot}8$) is around 200, method (*b*) and 700, method (*a*).

The similarity of the protein : degraded carrageenan ratio at saturation value and in the digest for maximum inhibition, supports the idea of a system wherein the degraded carrageenan and protein molecules react in small units. The marked difference, on the other hand, between the corresponding protein : undegraded carrageenan ratios suggests that one undegraded carrageenan molecule can effectively cover and protect from digestion many protein molecules.

It must be pointed out that although undegraded carrageenan can, in the present type of experiment, be shown to inhibit peptic activity more effectively, such a difference may not be realized clinically. The much more rapid and easy dissolution and greater solubility, coupled with the possibility of using greater dosage of degraded carrageenan would here assume greater importance. Indeed the time taken for the undegraded molecule to dissolve in gastric juice would result in a large amount of its potential activity being unavailable.

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