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Effect of Sulphur Dioxide on the Activity of Trypsin

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ABSTRACT

The effects of sulphur(IV) oxospecies [S(IV)] on the rate of hydrolysis of N-*benzoyl*-D,L-*arginine*-p-*nitroanilide by bovine trypsin are described. The enzyme is partly inhibited in a biphasic process: a rapid reduction of activity in a process capable of being saturated by S(IV) is followed by a change, on the timescale of hours, whose rate is independent of the concentration of S(IV).*

INTRODUCTION

Trypsin is an important enzyme in protein digestion as it not only exhibits direct hydrolytic action upon dietary proteins but is also responsible for the activation of other proteolytic enzymes of the pancreas. Trypsin inhibitors thus severely limit protein digestion.

Sulphur(IV) oxospecies [S(IV)] may be present in food as free additive (SO_2, HSO_3^-, SO_3^{2-}, $S_2O_5^{2-}$ species) or in reversibly bound form as hydroxysulphonate adducts of carbonyl components of the food. The latter are relatively stable in the acid environment of the stomach but the alkaline environment of the duodenum causes the release of such reversibly bound S(IV) (Gibson & Strong, 1976). The higher pH also ensures that the S(IV) is present in the form of SO_3^{2-} ion which is an effective nucleophile. Sulphite ion is therefore likely to come into contact with trypsin in the duodenum.

Sulphite ion has been shown to cleave five out of six disulphide bonds of trypsin (Cecil & Wake, 1962). It is therefore surprising to find that

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Moutonnet (1967) reports that the addition of S(IV) at levels of up to 250 mg potassium disulphite per gram of substrate has no effect on trypsin activity. In view of these seemingly contradictory results it was felt that a further investigation of the effects of S(IV) on trypsin activity was warranted.

EXPERIMENTAL

The assay of trypsin activity involved the use of *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) which is cleaved by the enzyme to form *N*-benzoyl-D,L-arginine and *p*-nitroaniline. Assay mixtures were buffered at pH 7·8 with triethanolamine (TEA) buffer. The substrate (BAPNA), *p*-nitroaniline, TEA hydrochloride and trypsin were obtained from Sigma Chemicals Ltd. Trypsin was bovine pancreatic described as dialysed and lyophilised and had been treated with TPCK. All other reagents were obtained from BDH Chemicals Ltd and, where possible, were of AnalaR grade.

Stock solutions of substrate were prepared by dissolving a known weight of BAPNA in DMSO + methanol (2 + 3 v/v) solvent. The stock buffer medium consisted of TEA hydrochloride (0·2 M) whose pH had been adjusted to pH 7·8 with NaOH (2 M). When it was desired to test the effect of S(IV) on the activity of trypsin, weighed amounts of sodium disulphite were added to the TEA buffer before final pH adjustment. All solutions contained EDTA (1 mM) to minimise metal-catalysed autoxidation of S(IV). A stock solution of trypsin was prepared by dissolving trypsin (approximately 2 mg) in HCl (1 mM, 10 ml). The solution was stored at 4°C for up to 2 weeks. For use, the stock solution was diluted with water to obtain a preparation with an appropriate enzyme activity.

All assays were carried out at 25 ± 0·1°C and prior to each assay all reagents were allowed to come to thermal equilibrium at this temperature. For analysis, buffer (3·0 ml) and BAPNA solution (0·8 ml) were placed in a 1 cm cell in the thermostatted cell block of a Cecil 292 UV/VIS spectrophotometer and the reaction started by the addition of the enzyme preparation (0·2 ml). The cell was stoppered and the contents rapidly mixed by inverting the cell and the absorbance at 405 nm of the mixture recorded as a function of time. A blank was prepared in the same way but replacing the enzyme preparation by water (0·2 ml). The concentration of the enzyme preparation was adjusted to give a maximum initial rate of change of absorbance of the order of 0·05 units min^{-1}.

The effect of S(IV) on the activity of trypsin can only be investigated meaningfully once it has been shown that S(IV) does not modify the absorbance of *p*-nitroaniline. To test for any such effect, an aliquot (1 ml) of a solution of *p*-nitroanaline (0·17 mM in DMSO + methanol, 2 + 3 v/v) was

mixed separately with TEA buffer containing S(IV) (52 mM) to give a final volume of 10 ml. The mixture was held at 25°C while its absorbance at 405 nm was measured as a function of time.

The concentration of S(IV) in assay mixtures was found using the spectrophotometric method of Humphrey *et al.* (1970) involving the use of 5,5′-dithiobis(2-nitrobenzoic acid). This technique was calibrated using iodimetrically standardised solutions of S(IV).

RESULTS AND DISCUSSION

Choice of assay procedure

The most sensitive assays of trypsin activity involve the measurement of its rate of hydrolysis of specific substrates, including proteins and a variety of 'synthetic substrates'. Proteins which have been used for the determination of trypsin activity include casein, fibrinogen, fibrin and denatured haemoglobin. Moutonnet (1967) examined the effect of S(IV) on the ability of trypsin to cleave proteins in skim milk powder, gelatin or gluten. The main problem caused by the use of realistic protein substrates is the ability of S(IV) to modify the substrate by cleavage of disulphide bonds, perhaps rendering the protein more susceptible to enzymic hydrolysis.

The most important synthetic substrates are esters and amides. Trypsin is a good catalyst for the hydrolysis of substrates such as α-benzoyl-L-arginine methyl ester and the enzyme can therefore be assayed for its esterase activity. The main disadvantage of this technique lies in the possibility that the inhibition of esterase activity of proteases need not parallel the inhibition of their proteolytic or biological activity. The most useful synthetic substrates for trypsin for this investigation are based on its specificity for peptide bonds adjacent to arginine residues where the labile amide link is to a chromogenic group such as *p*-nitroaniline which has $\lambda_{max} = 405$ nm and $E_{max} = 9900\ M^{-1}\ cm^{-1}$. A commercially available substrate with such groups is BAPNA. Preliminary experiments have shown that at the highest concentration of S(IV) used in the assay mixture (47 mM) the additive has no effect on the absorbance of *p*-nitroaniline and does not give rise to a measurable release of *p*-nitroaniline from BAPNA over the time-course of trypsin assays (10 min).

Preliminary assessment of assay procedure

Numerous workers comment that Ca^{2+} ions are necessary to observe maximum trypsin activity, one role of the ion being to stabilise the enzyme

against autolysis (Bier & Nord, 1951). The addition of 20 mM Ca^{2+} ion to trypsin assays is widely recommended (Walsh, 1970; Keil, 1971; Rick, 1974). The solubility of $CaSO_3$ is of the order of 0·5 mM (Linke, 1958) and it is expected, therefore, that assay mixtures containing 20 mM Ca^{2+} ion and S(IV) at concentrations up to 50 mM will form precipitates of $CaSO_3$ and any apparent effects of S(IV) on enzyme activity could be the result of its effect on the Ca^{2+} ion component rather than on the enzyme itself. In order to evaluate the importance of the Ca^{2+} ion, assays were carried out with and without added $CaCl_2$ at the recommended concentration with [BAPNA] = 0·88 mM. In the presence of Ca^{2+} ion an initial rate of $3{\cdot}41 \pm 0{\cdot}02$ μM min^{-1} was observed compared with an initial rate of $3{\cdot}42 \pm 0{\cdot}02$ μM min^{-1} in its absence. The results represent means $\pm$ standard deviations from four independent runs in each case and the means are clearly indistinguishable at a high level of significance. Blank runs with and without Ca^{2+} ion but containing no enzyme gave the same absorbance which remained constant over the period of measurement. Calcium ion was therefore omitted from subsequent assays.

It is desirable that the substrate in any enzyme assay saturates the enzyme. In order to confirm that the initial rate was independent of the concentration of BAPNA the assay was carried out using [BAPNA] = 0·88 and 1·74 mM. Under these conditions initial rates of $3{\cdot}83 \pm 0{\cdot}02$ and $3{\cdot}82 \pm 0{\cdot}02$ μM min^{-1}, respectively, were obtained, confirming that the substrate saturates the enzyme when its concentration is greater than 0·88 mM.

Effect of S(IV) on initial rate

The effect of S(IV) on the initial rate of hydrolysis of BAPNA was determined as a function of concentration of S(IV) and time. The effect of concentration is summarised in Table 1 for assays on enzyme/S(IV) mixtures which had been kept for 1 h at 25°C prior to analysis. It is evident that reaction of the enzyme with a relatively dilute solution of S(IV) (0·8 mM)

TABLE 1
Effect of S(IV) on the Activity of Trypsin. Reaction Conditions: [BAPNA] = 0·88 mM; pH, 7·8; 25°C

[S(IV)] (*mM*)	*Initial rate* (μM min^{-1})
0·0	4·78
0·8	3·07
23·5	2·70
43·9	2·70

leads to a very significant reduction in activity of 36%, whilst increase in concentration by a factor of 55 results in only a small further lowering of enzyme activity to give a total reduction of activity of some 39% of the original value. The inhibitory effect was found to be time-dependent. When [S(IV)] = 47 mM it is found that 46% of the change in activity observed when the enzyme is allowed to react with S(IV) for 1 h occurs within the first 5 min of reaction. After this initial change in activity there is a time-dependent loss which appears to continue well beyond 1 h. The observation that the initial rate of hydrolysis in enzyme/S(IV) mixtures which have been kept for 1 h is little affected by a 55-fold increase in S(IV) suggests that the time-dependent reaction of trypsin with S(IV) is of zero order with respect to S(IV). No reduction of activity was observed in the absence of S(IV) and the additive appears to be essential to the change. Two mechanisms for the involvement of S(IV) can be envisaged.

(1) The additive, in a fast reaction with trypsin, leads to an unstable structure which retains some enzyme activity but which subsequently denatures slowly or undergoes autolysis.
(2) Trypsin could be converted slowly from one active form to another. If the new form reacts more readily with S(IV) than the other and the reaction with S(IV) is relatively fast then the overall process will be of zero order with respect to S(IV).

Commercial bovine trypsin which was used in this study contains several different active forms of the enzyme which arise from autolysis (Schroeder & Shaw, 1968; Smith & Shaw, 1969). They differ from one another by the number of places in which the polypeptide chain is cleaved and in their specific activity towards BAPNA (Keil, 1971). Autolysis takes place in the absence of Ca^{2+} ion but there was no evidence of this over the timescale of the experiments in this work. It is possible, however, that S(IV) may assist conversion by altering the tertiary structure through cleavage of disulphide bonds. Cecil & Wake (1962) showed that whereas intermolecular disulphide bonds will invariably react with sulphite ion, intramolecular disulphide bonds do so only in exceptional cases or in the presence of denaturing agents. While the trypsin molecule derived directly from trypsinogen contains only intramolecular disulphide bonds, the other active forms can be considered to contain varying numbers of intermolecular bonds. It is estimated that at pH 7·8 trypsin has a net positive charge of 6. The susceptibility of a molecule to disulphide cleavage increases with increasing positive charge (Cecil & McPhee, 1955; McPhee, 1956) and if any of these charges on trypsin are located near disulphide bonds then attack by sulphite ion will be enhanced.

It is not possible to estimate reliably the actual concentration of trypsin in

assay mixtures. A typical concentration of the enzyme, based on the amount of solid weighed, was of the order of 0·1 g litre^{-1} and assuming a molecular weight of 23 750 daltons for trypsin (Rick, 1974), the maximum possible concentration of the enzyme is of the order of 4·2 μM. Therefore, the concentration of S(IV) in the most dilute system studied (0·8 mM) represents a molar excess of S(IV) over trypsin of at least 190. It is reasonable to expect, therefore, that the mechanisms involved in the inhibition need not be limited by S(IV).

All experiments were carried out under saturating conditions of BAPNA which may not be appropriate for studies where the inhibition is competitive. The reaction of S(IV) with simple disulphides is reversible since a thiol group can displace a sulphite ion from the S-sulphonate. If the substrate helps to orientate the protein to reform disulphide bonds then the effect is not apparent at the lowest S(IV) concentration (0·8 mM) used since this already saturates the enzyme with inhibitor at a reaction time of 1 h. The possibility that large concentrations of substrate could reverse the inhibition is, of course, very relevant to the action of trypsin *in vivo* where it may come into contact with high concentrations of protein. The study of such an effect now requires a more realistic substrate than BAPNA.

CONCLUSION

Contrary to a previous report, it is evident that trypsin is partially inhibited by S(IV) in a biphasic process. The potential for the inhibition of trypsin *in vivo* needs to be assessed, particularly in view of there being a rapid process by which activity is lost.

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