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The Preparation and Properties of Cystine Esterase

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(Received 8 July 1960)

Hooper & Jessup (1959) showed that placenta contain separate enzyme systems destroying oxytocin and vasopressin. During the purification of the enzymes, it seemed desirable to replace the biological estimation of oxytocinase by a chemical method using synthetic substrates. Certain properties of oxytocinase were revealed by inhibitors (Hooper, 1959), and it was concluded that the enzyme is a peptidase with esterase activity. Attempts have been made to find a chemical method of assay based on the presumed esterase activity of oxytocinase. A variety of esters was incubated with crude preparations of the enzyme and an additional enzyme which hydrolysed esters

of cystine and of *S*-benzylcysteine, which has been called cystine esterase, was found. This report describes some of its specificities and its partial purification.

METHODS

Estimation of amino acids. Amino acid ester hydrochlorides were incubated with enzyme as described below. Samples equivalent to approx. 100 μ g. of original substrate were chromatographed on Whatman no. 1 paper with butan-1-ol-acetic acid-water (5:1:4, by vol.). After spraying with ninhydrin solution (0.1% in butan-1-ol-2% acetic acid) the colour was developed at room temperature for 1 hr., followed by heating at 80° for 5 min. Spots produced by amino acids were cut out, the colour was eluted with 3 ml. of propan-1-ol-water (1:1, v/v), and the extinction at 580 m μ measured. Determinations were done

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in duplicate or triplicate, and control samples of ester and known amounts of amino acids were chromatographed concurrently with the products of enzyme digestion.

Estimation of enzyme activity. The standard conditions used in most experiments were as follows: enzyme preparations were incubated at 37° with *S*-benzyl-L-cysteine ethyl ester hydrochloride (5.5 mg./ml.; 0.02M) in 0.1M-phosphate buffer, pH 7.7. Samples (20 μ l.) were withdrawn at intervals and the amount of *S*-benzyl-L-cysteine formed was estimated as described above.

Preparation of amino acid ester hydrochlorides. The ethyl ester hydrochlorides of the following amino acids were made according to the Fischer method: DL-alanine, L-arginine, L-aspartic acid, L-cystine, *S*-benzyl-L-cysteine, glycine, L-glutamic acid, L-histidine, DL-leucine, L-lysine, DL-methionine, DL-phenylalanine, DL-serine, DL-threonine and L-tyrosine. *S*-Benzyl-DL-cysteine was prepared by treating the L isomer with acetic anhydride (Wood & du Vigneaud, 1939) and was esterified by the above-described method.

Buffers. Phosphate buffers (NaH_2PO_4 - Na_2HPO_4) were used to maintain hydrogen-ion concentrations between pH 5.5 and 8.5. Below pH 5.5 acetate buffers (sodium acetate-acetic acid) were employed, and above pH 8.5 borate buffers (sodium borate-boric acid) were used. All buffers were used at 0.1M-concentrations unless stated otherwise.

RESULTS

Partial purification of enzyme. The enzyme was obtained from human placenta; all operations were done near +2°. Tissue was ground with sand and 0.25M-sucrose solution (1 ml./g. wet wt.). The supernatant obtained after centrifuging the homogenate at 30 000g for 30 min. was made 45% saturated with respect to ammonium sulphate by addition of 0.8 vol. of saturated ammonium sulphate solution. After the solution was stirred for 30 min. the precipitate was sedimented and dis-

carded. The supernatant was then made approx. 65% saturated by addition of 1 vol. of saturated ammonium sulphate solution. The precipitate was collected and the supernatant discarded. The precipitate was dissolved in the minimum volume of 0.01M-phosphate buffer, pH 8.0, and dialysed against similar buffer to remove ammonium sulphate. The solution, which had been clarified by centrifuging, was chromatographed on an hydroxyapatite column (Tiselius, Hjertén & Levin, 1956) equilibrated with 0.01M-phosphate buffer, pH 8.0. Proteins were eluted by a stepwise increase in molarity of phosphate buffers, pH 8.0. The molarity of the buffers used and the elution pattern of protein measured at 280 m μ are shown in Fig. 1. The eluate containing the major peak of enzyme activity was dialysed against water and freeze-dried. The powder formed a pale-pink solution when dissolved in water.

pH of optimum enzyme activity. The variation of enzyme activity with hydrogen-ion concentration is shown in Fig. 2. The non-enzymic hydrolysis of the substrate at the different pH values selected was determined by incubation in the absence of enzyme; the corrections obtained have been applied to Fig. 2. The activity curve shows an optimum pH of 7.7-7.8.

Reaction with inhibitors. The crude preparation obtained by ammonium sulphate precipitation possessed both cystine-esterase and oxytocinase activities. The preparation was treated for 15 min. at pH 7.7 with enzyme inhibitors over a range of concentrations before addition of *S*-benzyl-L-cysteine ethyl ester hydrochloride or oxytocin (Pitocin; Parke, Davis and Co. Ltd.). Residual oxytocin was assayed on the isolated uterus of the rat and converted into oxytocinase units by means

Fig. 1. Chromatography of impure enzyme on hydroxyapatite column (25 cm. $\times$ 2 cm.); 5 ml. fractions were taken. Continuous line is protein concentration measured at 280 m μ . Fractions were incubated with *S*-benzyl-L-cysteine ethyl ester hydrochloride for 4 hr. Amino acid formed was developed with ninhydrin and extinction at 580 m μ was measured (broken line). Molarity of eluting buffers was increased where indicated by arrows.

Fig. 2. Activity of enzyme as a function of hydrogen-ion concentration. Enzyme was incubated with substrate (0.02M) for 4 hr. at the pH values indicated; buffers were 0.1M. Liberated *S*-benzyl-L-cysteine was developed with ninhydrin as described in the Methods section.

Fig. 3. Inhibition curves of oxytocinase treated with Cu^{2+} , Ag^+ or Zn^{2+} ions. Enzyme was treated with inhibitors for 15 min. at pH 7.7 before addition of oxytocin. Reaction mixture was then incubated for 30 min. at 37° . Residual oxytocin was assayed on isolated rat uterus and converted into enzyme units as described by Hooper (1959). ■, Cu^{2+} (1 mm); ▲, Ag^+ (0.1 mm); ●, Zn^{2+} (0.1 mm).

of a reference graph (Hooper & Jessup, 1959). Cystine-esterase activity was also measured. Fig. 3 shows the effect of three inhibitors on oxytocinase; Cu^{2+} (1 mm), Ag^+ (0.1 mm) and Zn^{2+} (0.1 mm) ions caused virtually complete inhibition of oxytocinase. Cystine esterase was unaffected by these three metal ions at 1 mm-concentrations.

Specificity. After incubation for 18 hr., the ethyl ester hydrochlorides of the following amino acids were not hydrolysed to any detectable extent by the enzyme: DL-alanine, L-arginine, L-aspartic acid, glycine, L-glutamine, L-histidine, DL-leucine, L-lysine, DL-methionine, DL-serine, DL-threonine and L-tyrosine. Under the same conditions, *S*-benzyl-L-cysteine ethyl ester hydrochloride was converted completely into a substance chromatographically indistinguishable from the parent amino acid. L-Cystine diethyl ester hydrochloride gave a number of spots, the predominant one corresponding to L-cystine.

DISCUSSION

The estimation of amino acids as described here is not the most sensitive of methods. Estimation by development with Moore & Stein (1948) reagent after elution of amino acids from chromatograms was approximately three times more sensitive. When determinations were made on products of enzyme digestion, however, blank measurements with the Moore & Stein reagent were high and

variable. The less-sensitive but more-reproducible method of elution of the developed colour was preferred.

S-Benzyl-L-cysteine ethyl ester hydrochloride was chosen as substrate for several reasons. First, the production of several spots by enzymic digestion of L-cystine diethyl ester hydrochloride suggested that disulphide interchange, analogous to that described by Ryle & Sanger (1955), occurred between L-cystine and the ester. Secondly, *S*-benzyl-L-cysteine ethyl ester hydrochloride is relatively easy to prepare in a pure form whereas L-cystine could not be obtained free from L-tyrosine, and the corresponding ester was not chromatographically pure. *S*-Benzyl-L-cysteine ethyl ester hydrochloride proved to be relatively stable under the conditions of incubation and corrections for non-enzymic hydrolysis were small.

In addition to undergoing hydrolysis by homogenate of human placenta, *S*-benzyl-L-cysteine ethyl ester hydrochloride was rapidly hydrolysed by homogenates of rat liver, kidney, heart, lung and spleen, and to a lesser extent by those of uterus and brain. Esterases are widely distributed (Ammon & Jaarma, 1951), and many do not appear to have high substrate specificities. It is possible that several enzymes may be responsible for the hydrolysis of *S*-benzyl-L-cysteine ethyl ester hydrochloride by tissue homogenates. During the preliminary stages of purification, advantage was taken of the fact that cystine esterase was precipitated along with oxytocinase during the ammonium sulphate-precipitation step. It is not possible therefore to give an indication of the purification achieved, since in the early purification steps the enzyme was not estimated by hydrolysis of the ester.

Cystine esterase is not identical with oxytocinase. First, with an impure preparation containing both activities, oxytocinase was inhibited by concentrations of metals ineffective on cystine esterase. Secondly, oxytocinase is more heat-labile than is cystine esterase, the latter remaining partially active when heated at 60° for 5 min. whereas oxytocinase activity was not detectable after such treatment. Also, when chromatographed on a hydroxyapatite column the peak of cystine-esterase activity did not coincide with any one of the peaks of oxytocinase activity.

Cystine esterase shows an interesting specificity. In the configuration $\text{H}_2\text{N}\cdot\text{CH}(\text{CH}_2\cdot\text{S}\cdot\text{R})\cdot\text{CO}_2\text{R}'$ the presence of the group $\text{R}\cdot\text{S}\cdot\text{CH}_2\cdot$ appears to be necessary for enzymic fission of the hydrolysable bond at R' . The substrate specificity of the enzyme has not yet been investigated in any great detail. Preliminary results suggest that the nature of R' is not highly specific since methyl, ethyl and benzyl esters are hydrolysed. In experiments with

S-benzyl-DL-cysteine ethyl ester hydrochloride as substrate, the D isomer did not act as a competitive inhibitor. The specificity of cystine esterase is different from that of chymotrypsin and trypsin. Chymotrypsin requires the presence of an aromatic group for activity, and the hydrolysis of L-tyrosine ethyl ester may be used as a method of assay (Balls & Jansen, 1952). Trypsin requires the presence of basic groups and rapidly hydrolyses L-lysine ethyl ester (Dixon & Webb, 1958). In the conditions described earlier, cystine esterase did not hydrolyse either of these esters to any detectable extent.

SUMMARY

1. An enzyme occurring in human placenta hydrolyses esters of cystine and *S*-benzylcysteine more rapidly than it does those of other amino acids. The enzyme has been partially purified by ammonium sulphate precipitation and chromatography on a calcium phosphate column.

The skilled technical assistance of Mrs M. Gordon and Mr J. Caird is gratefully acknowledged.

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Studies in the Biochemistry of Micro-organisms

108. METABOLITES OF *ALTERNARIA TENUIS* AUCT.: THE BIOSYNTHESIS OF TENUAZONIC ACID*

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(Received 8 August 1960)

Alternaria tenuis Auct., when grown in surface culture on Czapek–Dox medium, has been shown to produce several new metabolites. From the mycelium, Raistrick, Stickings & Thomas (1953) obtained alternariol and alternariol methyl ether, and showed the structure of alternariol to be 3:4':5-trihydroxy-6'-methyldibenzo- α -pyrone. From the culture filtrates of various strains, Rosett, Sankhala, Stickings, Taylor & Thomas (1957) isolated several new substances of unknown structure. One of these, tenuazonic acid, was later shown by Stickings (1959) to be 3-acetyl-5-sec.-butyltetramic acid (I). Thomas (1959, 1961) has recently shown by tracer studies that the carbon skeleton of alternariol is derived from acetate, and has pointed out that it could be obtained by head-to-tail condensation of acetate units, with no necessity for oxidation or reduction steps. Stickings (1959) suggested that tenuazonic acid might be

synthesized in the fungus from one molecule of L-isoleucine and two molecules of acetate, also without any oxidation or reduction steps, and we now report evidence which supports this proposal. A preliminary account of part of this work has been given (Stickings & Townsend, 1960).

A strain of *A. tenuis*, catalogue no. 628, was chosen for this investigation, since it had already been shown (Rosett *et al.* 1957) to give a good yield of tenuazonic acid but none of the other products. In order to decide the most suitable time for addition of tracer acetate, the growth of *A. tenuis* was studied over a period of 6 weeks. It was shown that, in undisturbed surface culture, the concentration of tenuazonic acid increased rapidly from a low value on the fifth day to a maximum on the thirteenth to the fourteenth days. Other aspects of the growth and production curves are discussed later.

Sodium [$1\text{-}^{14}\text{C}$]acetate was therefore added on the fifth day to growing cultures, and these were

* Part 107: Birkinshaw & Samant (1960).