METHODS FOR THE DETECTION AND QUANTITATION OF ANGIOTENSIN CONVERTING ENZYME INHIBITORS IN FERMENTATION BROTHS†

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Two procedures are described for the detection of inhibitors of angiotensin converting enzyme (ACE). The first is a new agar-plate method useful as a screening tool. Two ACE inhibitors produced by culture A58365 were discovered using this plate test. The second method is a modification of a previously reported spectrophotometric procedure. Both procedures utilize *p*-nitrobenzyl-oxycarbonylglycyl-(*S*-4-nitrobenzo-2-oxa-1,3-diazole)-L-cysteinylglycine as substrate.

Angiotensin converting enzyme, ACE, (dipeptidyl carboxypeptidase EC 3.4.15.1) catalyzes the release of dipeptide from the carboxyl terminus of angiotensin I to yield the octapeptide angiotensin II, a potent vasoconstrictor. The same enzyme inactivates the vasodilator, bradykinin, by the hydrolytic release of one or more carboxyl-terminal dipeptide residues. Although the use of ACE inhibitors is well established in treatment of hypertension and congestive heart failure, there were no known fermentation derived inhibitors of ACE at the time this screening program was initiated. There is, however, ample precedent for screening for enzyme inhibitors in fermentation broths.[1,2] Since the initiation of this project several interesting ACE inhibitors from fermentations have been reported.[3~6]

Materials and Methods

Reagents

Captopril (1-[(2*S*)-3-mercapto-2-methylpropionyl]-L-proline)[7] was a gift from E. R. Squibb & Sons. Enalapril [*N*-(1-carboxy-3-phenylpropyl)-L-alanyl-L-proline][8] was a gift from Merck Sharp & Dohme. *p*-Nitrobenzyl-oxycarbonylglycyl-(*S*-4-nitrobenzo-2-oxa-1,3-diazole)-L-cysteinylglycine (NBGCG)[9] was synthesized in the Lilly Research Laboratories.

Enzyme Preparation

Crude soluble ACE was prepared from acetone powder of rabbit lung (Pel Freeze Biologicals) by blending 6 g of powder in 60 ml of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 6.5) containing 100 mM NaCl,[10] 200 μM $CoCl_2$,[11,12] and 30 mM octylglucoside.[13] The $100,000\times g$ (1 hour, 4°C) supernatant was dialyzed against 20 mM HEPES buffer (pH 6.5) containing 100 mM NaCl and 200 μM $CoCl_2$ to remove the octylglucoside. This preparation is stable under refrigeration for several months. Protein determinations were done by the method of LOWRY *et al.*[14]

Plate Assay for Detecting ACE Inhibitors

The agar plate assay involved applying the putative inhibitor to an enzyme-containing layer,

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Fig. 1. ACE inhibitor assay plate after treatment with 0.1 N NaOH.

Photographed through a Wratten #36-B420 Eastman Kodak filter. The zone of inhibition (22 mm diameter) corresponds to 500 ng of enalapril on a 7-mm filter paper disc (sample solution, 10 μg/ml).

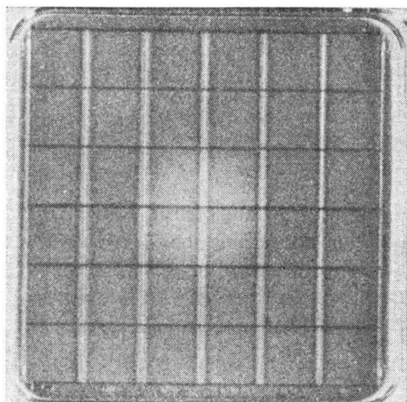
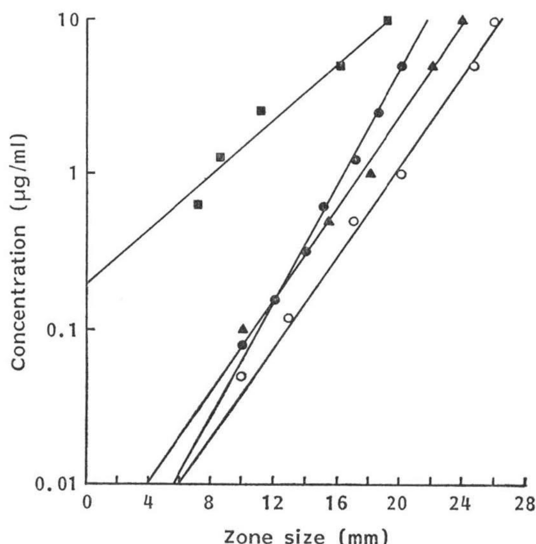


Fig. 2. Inhibitor concentrations *versus* zone diameter plot.

Concentration values represent the concentration of the solutions used to saturate 7 mm filter paper discs.

■ Captopril, ● enalapril, ○ A58365A, ▲ A58365B.



allowing time for diffusion, and then overlaying with a substrate-containing layer. The lower layer contained 1% agarose (FMC Corp.) in 20 mM HEPES buffer (pH 6.5), 100 mM NaCl, 200 μM $CoCl_2$, and crude ACE at a final concentration of 400 μg protein/ml agarose. Fermentation broths were either dispensed (50 μl) into 6 mm wells cut in the agar or dried on 7 mm filter paper discs before being placed on the agar surface. Ten ml of agarose are required per 100 cm^2 plate for each of the two layers. After a 2-hour diffusion time at room temperature, filter paper discs were removed and the enzyme layer was overlaid with the second layer containing 1% agarose, 20 mM HEPES buffer (pH 6.5), 100 mM NaCl, 200 μM $CoCl_2$ and NBGCG at 200 μg/ml (350 μM). Following a 2-hour incubation at 37°C the plates were flooded with 0.1 N NaOH for 10 minutes after which the solution was poured off. Zones of inhibition were initially seen as colorless zones on an amber background. The colorless zones turned pink after 30 minutes and faded completely in two hours.

Measurement and recording of zones of inhibition were facilitated by viewing plates through a blue filter (Wratten #36-B420 Eastman Kodak). Under these conditions, black and white photographs showed white zones on a black background, as seen in Fig. 1.

Spectrophotometric Assay for ACE Inhibitors

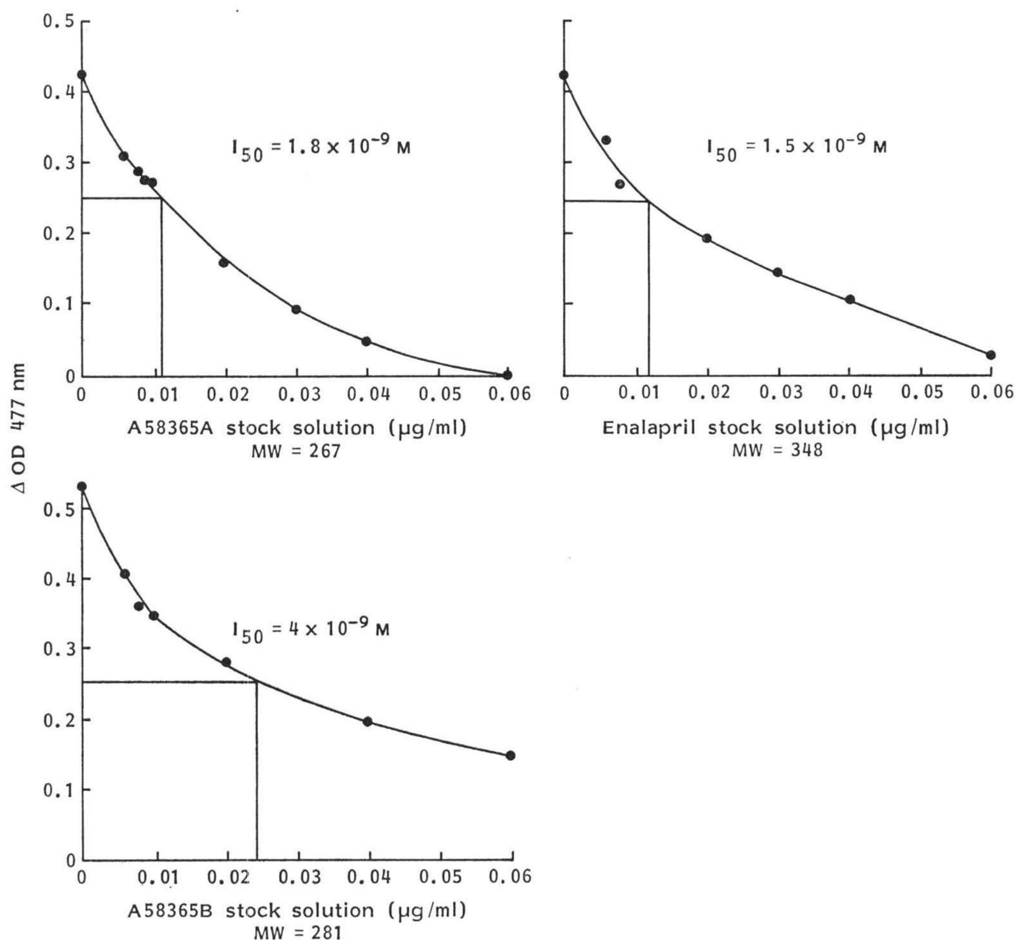
Enzyme reactions for I_{50} determinations were initiated by the addition of 50 μl of crude ACE (above) at 20 mg protein/ml to 2.0 ml of 20 mM HEPES buffer (pH 6.5) containing 100 mM NaCl, 200 μM $CoCl_2$, 455 μM 4,4′-dithiodipyridine, 18 μM NBGCG substrate and 100 μl of inhibitor solution (all at 37°C). After a 3-minute incubation time (water bath, 37°C), the reaction was terminated by the addition of 100 μl of enalapril[13] (10 μg/ml). The reaction is linear for 10 minutes. Absorbance was read at 477 nm *versus* a blank consisting of a complete reaction mixture as outlined above, to which enalapril was added before addition of the enzyme. The NBGCG substrate has an absorption maximum at 425 nm. The product of the reaction has a maximum absorbance at 460 nm. Maximum selectivity was achieved by measurement at 477 nm.

Results and Discussion

Approximately 1,600 fermentation broths were screened using the plate test before culture

Fig. 3. I_{50} values for A58365 ACE inhibitors from 3-minute reactions at 37°C with 50 µl of crude ACE at 20 mg protein/ml with 2.0 ml of 18 µM NBGCG in 20 mM HEPES, pH 6.5 containing 100 mM NaCl, 200 µM $CoCl_2$ and 455 µM 4,4'-dithiodipyridine.

The reactions were stopped at 3 minutes by the addition of 100 µl of enalapril at 10 µg/ml.



A58365[15,16] was found. The original fermentation broth produced an 18-mm zone which is now known to represent less than 1 µg/ml of A58365 ACE inhibitors.

The plate test is semiquantitative, giving measurable zones for A58365A, A58365B, captopril, and enalapril in a concentration range from 0.1 µg/ml to 10 µg/ml which is equivalent to 5 ng of compound per filter paper disc at the lower concentration (Fig. 2). Consequently, the sensitivity of this test corresponds to levels of compounds commonly found in fermentation broths. The inclusion of cobalt (see Experimental section) served to significantly increase the activity of the enzyme.[11,12]

The difference in the slopes (Fig. 2) between captopril and the other inhibitors is not understood. It is interesting, however, that captopril is the only one of the four inhibitors that contains a sulfhydryl group.

The plate test is compatible with paper and cellulose thin layer chromatograms and was employed to monitor purification of ACE inhibitors.[15,16]

The relative potency of purified A58365 factors was measured spectrophotometrically. I_{50} values

found were: (Fig. 3)

A58365A: 1.8×10^{-9} M, A58365B: 4×10^{-9} M, enalapril: 1.5×10^{-9} M.

A direct correlation between the I_{50} values and the *in vivo* activity of the A58365 factors has not been established.

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