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AFFINITY PURIFICATION OF ENDOTHIA PROTEASE WITH A NOVEL RENIN INHIBITOR, SQ 32,970

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A novel tripeptidic renin inhibitor is described, SQ 32,970, that will potently inhibit endothia protease. This inhibitor can be coupled to Sepharose and will allow the affinity-purification of endothia protease in one step to greater than 95% purity as measured by SDS PAGE. The purified endothia protease cleaves the Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu substrate at the Phe-Nph bond with a K_{cat}/K_m of 7445 ($s^{-1}mM^{-1}$) at pH 3.1 and 4057 ($s^{-1}mM^{-1}$) at pH 6.0. Affinity purified endothia protease can be crystallized in the pH range in which it is enzymatically active and can be inhibited by renin inhibitors.

Endothia protease is an aspartyl protease produced by the fungus *endothia parasitica* and has been commonly referred to as endothiapepsin. Endothia protease has been crystallized and its three dimensional structure determined by X-ray diffraction (1). Endothia protease has been recently reported to bind several peptidic renin inhibitors with potencies equivalent to those obtained with renin and can co-crystallize with these inhibitors (2). X-ray diffraction studies with these enzyme-inhibitor complexes revealed a hydrogen bonding scheme that may be relevant to human renin. The molecular modeling of human renin based on endothia protease may allow a better understanding of the molecular interaction of renin with renin inhibitors. The renin inhibitors described to date that inhibit endothia protease with high potency are hexapeptide structures or larger that interact with subsites S_4 through S_2' in the active site of both renin and endothia protease (3). In this report, a novel tripeptidic renin inhibitor, SQ 32,970, is described that is truncated with respect to previously described renin inhibitors and potently inhibits both endothia protease and human renin. SQ 32,970 can be linked to Sepharose to allow a single-step purification of endothia protease from a commercial preparation. The affinity-purified enzyme is greater than 95% pure and suitable for crystallization.

Methods

Endothia protease was obtained as an impure extract from Pfizer Chemical Division, Milwaukee, WI and sold under the brand name SURECURD. Bovine

0006-291X/89 $1.50

chymosin, porcine pepsin and bovine cathepsin D were purchased from Sigma Chemical Co., St. Louis, MO. Human kidney renin was obtained from Dr. E. Haas at Mt. Sinai Medical Center, Cleveland, OH. Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu was custom synthesized by BACHEM, Torrance, CA.

Endothia protease, chymosin, pepsin, and cathepsin D were assayed by the decreased absorbance observed at 300 nm upon cleavage of the Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu substrate at the Phe-Nph bond according to the method of Dunn et al. (4). Cleavage at this site was confirmed by HPLC and FAB-Mass Spectroscopy. Routinely, Endothia protease was assayed at pH 3.1 in 100 mM sodium formate. When indicated, this enzyme was assayed at pH 6.0 in 100 mM sodium phosphate. Renin was assayed in 100 mM TES containing 5 mM EDTA, 5 mM sodium tetrathionate 2 mM phenyl methyl sulfonyl fluoride, pH 7.0 at 37 °C for 30 minutes. Angiotensinogen from human plasma (0.5 μM) was used as a substrate and angiotensin I was detected by radioimmunoassay. Protein concentration was determined by coomasie blue staining with BIORAD kits.

SQ 32,970 was synthesized by coupling N-BOC-6-aminocaproyl-phenylalanyl-leucine to (1S,2R)-1-cyclohexylmethyl-2-hydroxy-2-(2-thiazolyl)ethylamine using the HOBT-WSC method (the water soluble carbodiimide (WSC) used in these experiments was 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide), followed by removal of the N-terminal BOC protecting group using ca. 4N HCl in ethyl acetate. N-BOC-6-aminocaproyl-phenylalanyl-leucine was prepared from Phe-Leu-OMe (synthesized by standard peptide coupling procedures) by acylation with N-BOC-6-aminocaproic acid. Saponification of the product methyl ester gave the dipeptide free acid. To prepare the thiazole containing intermediate, BOC-cyclohexylalaninal was condensed with 2-lithiothiazole which was generated by the metallation of thiazole using n-butyllithium in tetrahydrofuran at -78 °C. The desired 1S,2R diastereomer was obtained by column chromatographic separation from the less potent hydroxyl epimer. To liberate the free amine for coupling, the product was treated with HCl in ethyl acetate to remove the BOC protecting group. SQ 32,970 was coupled to activated CH-Sepharose at 25 °C via the hydroxysuccinimide functionality of the matrix and the primary amine of SQ 32,970. Fifteen gm of washed Sepharose was reacted with SQ 32,970 at a concentration of 1.3 mM in 0.1 M sodium acetate, pH 6.0 in 0.5 M NaCl for 60 min. Excess ligand was removed by washing with acetate buffer and the remaining reactive groups of the matrix were blocked with 0.1 M Tris pH 8.0 in 0.5 M NaCl.

Surecurd solution was diluted with 100 mM sodium formate, pH 3.1, to a protein concentration of 4.0 mg/ml and 10 ml was applied to an SQ 32,970-Sepharose column (10 ml bed volume). The column was washed with 3 bed volumes of 100 mM sodium formate, pH 3.1, containing 0.5 M guanidine-HCl and 1.0 M NaCl. An additional wash with 3 bed volumes of 10 mM Tris, pH 7.0, containing 0.5 M Guanidine-HCl and 1.0 M NaCl was performed before eluting the endothia protease with 10 mM Tris, pH 8.0 containing 1.0 M NaCl. Five-ml fractions were collected into tubes containing 1 ml of 100 mM sodium formate pH 3.1. Additional chromatography on DEAE-Sephacel (Pharmacia) was performed by applying the affinity-purified endothia protease in 50 mM sodium formate and eluting the enzyme with a linear gradient from 0.05 M to 0.8 M sodium formate.

Results

The hydrolysis of Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu was monitored by the shift in the UV scan and by HPLC as shown in Fig. 1. The absorbance maximum shifts from 280 nm to 270 nm during the first 12 minutes of hydrolysis at the enzyme concentration used. The decreased absorbance observed at 300 nm was used as the basis for determining endothia protease activity. A complete shift in absorbance was evident after 12 minutes and this corresponds to the 77% decrease in substrate as measured

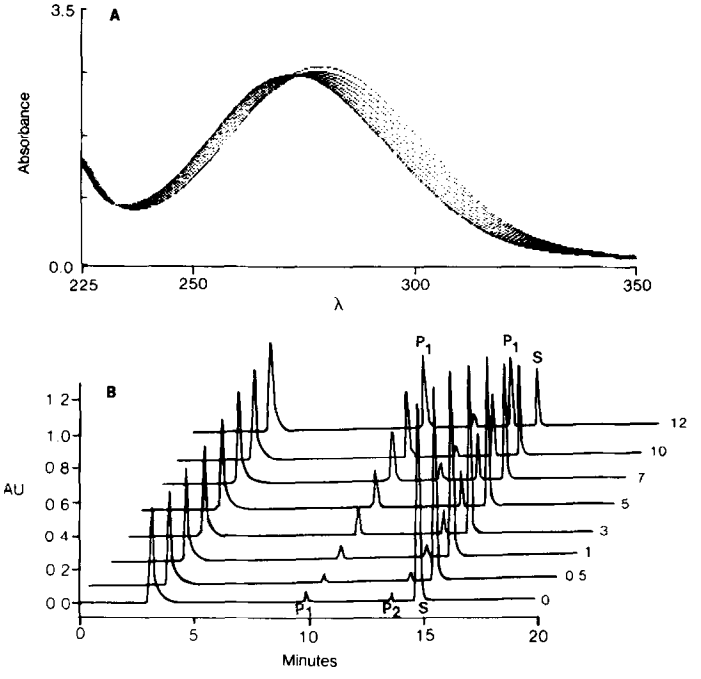
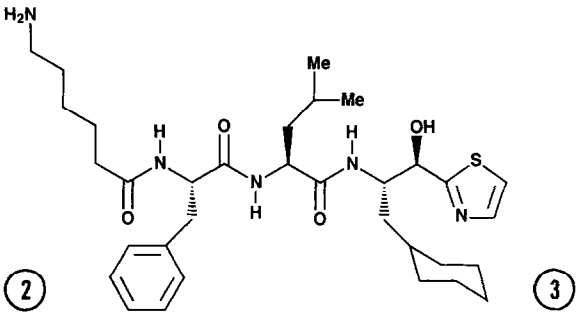


Figure 1. The spectrophotometric and HPLC assay of Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu by endothia protease. Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu (100 nM) was incubated with 2 μg of endothia protease in 100 mM sodiun formate buffer, pH 3.1. A shift in the UV absorbance maximum to the left is evident in Figure 1A where the sample was scanned every 2 minutes for a total of 12 minutes and the scans were overlayed. Figure 1B shows an overlay of the HPLC chromatograms of samples that were stopped at the indicated times by the addition of O.1 % TFA and acetonitrile and injected into a Waters HPLC system with a Vydac C18 RP column. The gradient went from 10 % acetonitrile to 60 % acetonitrile in 0.1% TFA over 20 minutes with absorbance monitored at 215 nm. The substrate (S) is shown at 14.7 min while P1 and P2 represent Lys-Pro-Ala-Glu-Phe, and Nph-Arg-Leu, respectively.

by HPLC (Fig. 1B). The overlay of HPLC chromatograms corresponding to different time points up to 12 minutes indicates that 2 main peaks appear upon hydrolysis of the octapeptide substrate (S) which has a retention time of 14.70 minutes. Peak 1 (P1) has a retention time of 9.80 min and corresponds to Lys-Pro-Ala-Glu-Phe while Peak 2 (P2) has a retention time of 13.55 min and corresponds to Nph-Arg-Leu. The identity of each peak was determined by FAB-Mass Spectroscopy. Additionally, P2 absorbed strongly at 254 nm as did the substrate indicating that these peptides contain Nph.

The structure of SQ 32,970 is shown in Fig. 2. This compound was probably conjugated to the hydroxysuccinimide functionality of Sepharose via its amino-terminal side chain. The affinity column was capable of binding 100 mg of endothia protease per 10 ml of packed gel. This corresponds to 236 nmoles of enzyme/ml gel. Complete coupling of SQ 32,970 to the resin would amount to 3,000 nmol/ml gel. Therefore, the amount of enzyme capable of binding to the column is significantly less than the theoretical capacity of the affinity column. The endothia protease eluting from the affinity column at pH 8.0 as a single peak of activity had a specific activity of 193.2



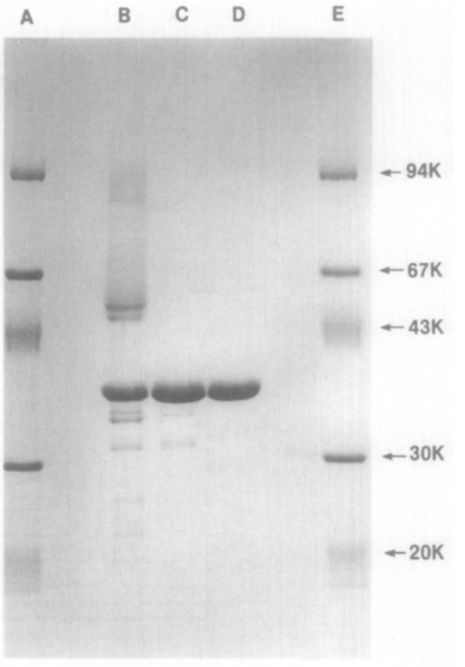


Figure 2. The structure of SQ 32,970.

Figure 3. SDS PAGE analysis of purified endothia protease. Samples of endothia protease (7.5 µg) at different stages of purification were applied to polyacrylamide gradient minigels (10%-20%). Molecular weight markers are shown in lanes A and E. Surecurd preparation is shown in lane B; endothia protease eluted from SQ 32,970-Sepharose is shown in lane C; and affinity-purified endothia protease after DEAE-Sepharose chromatography is shown in lane D.

µmol/min/mg which was a 2-fold increase in specific activity over the starting preparation. The recovery was at least 85% in each experiment performed. The affinity purified enzyme was analyzed by overloaded SDS PAGE as shown in Fig. 3 to visualize contaminating bands. Lane B shows the SURECURD material before application to the affinity column. Lane C shows the affinity-purified endothia protease. The dominant band is at 38,000, corresponding to the known molecular weight of endothia protease, and is 95% pure as determined by densitometric scans of each lane. The affinity purified material was further chromatographed on DEAE Sephacel to remove the minor contaminating bands seen in lane C. This preparation elutes in a linear salt gradient at 0.5 M NaCl and is 99% pure as seen in lane D. The low molecular weight contaminants seen in lane 3 are probably the result of autoproteolysis of endothia protease. If care is not taken to keep the enzyme at 4 °C these bands can be generated from purified enzyme such as seen in lane 4.

The K_m and K_{cat} values of endothia protease were determined with the octapeptide substrate at both pH 3.1 and pH 6.0. The Lineweaver-Burk plot shown in Fig. 4A indicates that endothia protease has a K_m of 8.3 ±1.5 µM and a K_{cat} value of 61.8 ± 4.8 s^{-1} (n=11) at pH 3.1. At pH 6.0, the enzyme has a K_m of 35 ± 5 µM and a K_{cat} value of 142 ± 9 s^{-1} (n=6). The K_{cat}/K_m ($s^{-1}mM^{-1}$) ratio is 7445 at pH 3.1 and

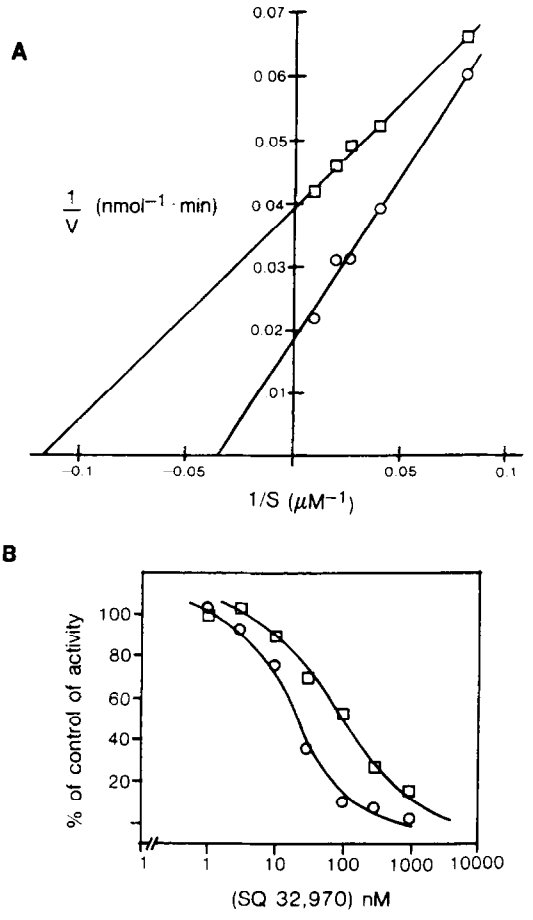


Figure 4. Effect of pH on kinetics and inhibition of endothia protease. The Lineweaver-Burk plot of affinity-purified endothia protease with Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu at pH 3.1 (□) and pH 6.0 (O) is shown in Figure 4A. The IC_{50} values for SQ 32,970 at pH 3.1 (□) and pH 6.0 (O) are shown in Figure 4B.

4057 at pH 6.0. The IC_{50} for SQ 32,970 is 100 nM at pH 3.1 and 20 nM at pH 6.0 (Fig. 4B). When corrected for the difference in K_m at these different pH values, the estimated K_i values are 20 nM and 10 nM at pH 3.1 and pH 6.0, respectively.

The potencies of SQ 32,970 against several other aspartyl proteases are shown in Table 1. Endothia protease and human renin are most potently inhibited by this compound (K_i = 20nM and 32 nM, respectively). Cathepsin D and pepsin are inhibited with moderate potency (73 nM and 113 nM, respectively). Chymosin is a weak inhibitor with a K_i value of 1130 nM. The K_m values reported for these enzymes are in good agreement with those reported by Dunn et al (4).

Discussion

SQ 32,970 has the lowest molecular weight of any renin inhibitor described to date that will also potently inhibit endothia protease. This inhibitor can also be effectively coupled to Sepharose to permit the affinity purification of endothia protease in one

Table 1. Inhibition of selected aspartyl proteases by SQ 32,970

	K_m (μM)	K_i (μM)
Chymosin (bovine)	189	1.130
Pepsin (porcine)	94	.113
Cathepsin D (bovine)	95	.073
Endothia protease	8	.020
Renin (human)	1.7	.032

The indicated proteases, except for renin, were assayed with Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu at pH 3.1. The K_m values were determined with at least 3 substrate concentrations and derived from Lineweaver-Burk plots. K_i values were estimated from IC_{50} values determined at a substrate concentration of 100 μM. Renin was assayed as described in the Methods section.

step. Enzyme of purity greater than 95% is obtained, depending on the extent of autoproteolysis that has occurred with the starting material and the yield is 85%. This affinity purification procedure takes advantage of the aminocaproic functionality of SQ 32,970 which couples with hydroxysuccinimide functionalities of the Sepharose. Endothia protease can be applied to the column at pH 3.1 and remains bound until it elutes at pH 8.0 where it is inactive and no longer has affinity for the inhibitor. The eluted enzyme was collected into fraction tubes containing sodium formate buffer at pH 3.1 to quickly restore the enzyme to a more favorable pH. A column prepared without the SQ 32,970 ligand but capped with Tris had no ability to bind endothia protease suggesting that purification is dependent on the presence of the inhibitor and was not working through other principles such as ion exchange. This is the first report of an affinity purification procedure for endothia protease using a renin inhibitor. The previously described procedure for purification of this enzyme was made 18 years ago by Whitacker (5) and employs ion exchange and gel filtration methods. In this report, the enzyme can be purified to greater than 95% purity in one step. Routine preparations made with the affinity method described here were suitable for crystallization using the batch method of Foundling (2). It is interesting to note that the SQ 32,970 column was not suitable for renin purification because the enzyme bound to the column but could not be eluted employing an extensive variety of eluting solvents. It is possible that some of the other mammalian enzymes such as cathepsin D or pepsin that are moderately inhibited by SQ 32,970 would bind and elute from the affinity resin described here. It is possible that the conditions to elute these enzymes may also be too severe to retain their activity.

The kinetic studies performed with purified endothia protease indicate that this enzyme is 50% less efficient toward the Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu substrate at pH 6.0 compared to pH 3.1. This is an important consideration when evaluating whether a renin inhibitor may also be an inhibitor of endothia protease because the inhibitor may carry a positive charge at pH 3.1 and appear to be inactive. At pH 6.0, the compound may be uncharged and active as an endothia protease inhibitor. Also,

endothia protease is easily crystallized in the pH 4.5 to pH 6.0 range. Therefore, it is important to establish the potency of inhibitors wirhin the same range in which they can be co-crystallized with the enzyme. The K_i values were estimated from the IC_{50} values rather than directly measured because very low substrate concentrations below the K_m cannot be effectively assayed. Therefore a K_i value cannot be determined from a Dixon plot in a meaningful manner. The characterization of the assay protocol is in good agreement with Dunn et al. (4) who demonstrated that the Phe-Nph bond is the primary site of hydrolysis. We demonstrated that the hydrolysis occurs at the same site in the peptide substrate and that the absorbance shift seen in the UV region coincides with the appearance of the peptide products as seen by HPLC. The absorbance in the HPLC experiments was monitored at both 215 nm and 254 nm but only the results obtained at 215 were shown since they clearly showed the appearance of both new products. At 254 nm the tripeptide Nph-Arg-Leu absorbed much more strongly. Also a small peak of unidentified nature appeared after 10 minutes of hydrolysis but accounted for less than 5% of the initial substrate concentration.

In conclusion, we have shown that endothia protease can be purified using a novel tripeptidic renin inhibitor affinity medium. The recovered enzyme is biologically active, has identical enzymatic properties compared to previous reports of endothia protease and can be crystallized in this form.

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