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Transition-State Affinity Purification of Proteases; The Preparation of an Argininal Affinity Resin for the Selective Binding of Trypsin-like Proteases

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An affinity resin containing Argininal (aldehyde) ligands is synthesized for the purification of proteases with trypsin-like specificity. The synthesis of arginine semicarbazone (Argal-SC) from Cbz-(ω-NO_2)-L-Arg, the joining of Argal-SC to an agarose matrix, and the removal of the semicarbazone function to form argininal is described. Argininal binds proteases by forming a hemiacetal with the serine nucleophile of the protease with high specificity due to the transition-state like features of the adduct. Thus the resin binds trypsin more strongly than resins that only associate by non-covalent interactions. The trypsin is eluted in quantitative yield by a semicarbazide containing buffer.

Peptidyl aldehydes have been shown to reversibly associate to serine and cysteine proteases as hemiacetal adducts with the nucleophilic amino acid residue of the protease active site (1-3). In the hemiacetal complex the carbonyl carbon of the aldehydic function has a tetrahedral (sp^3) configuration and thus mimics the tetrahedral intermediate and transition-state structures formed during the catalysis of substrates by these enzymes (1,4). Because of the transition-state like features of the complex, the peptidyl aldehydes show a high affinity and specificity for association, in agreement with the prediction of a high affinity for the binding of a transition-state analog to an enzyme (5,6). For example, whereas the non-covalent dissociation equilibrium constants for small peptide substrates to protease enzyme have K_S values of 10^{-4} to 10^{-2} M, the dissociation constants for peptide aldehyde analogs are two to four orders of magnitude smaller (1,4,7). Accordingly, peptidyl aldehydes should make a highly specific ligand for affinity chromatography. The development of selective high affinity ligands for protease enzyme purification would make an

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important contribution towards the characterization of proteases present in low concentrations, but required for essential cellular and physiological processes (8). In this paper we describe the synthesis and utilization of an Argininal affinity resin selective for proteases of trypsin-like specificity.

Experimental Section.

Synthesis of Cbz-(ω-NO_2)-L-Arginine Semicarbazone (Cbz-(NO_2)-Argal-SC).[1] The synthesis was similar to that of Shimizu et al. (9), with modifications as described: To a solution of 2.02 g (5.72 mMole) of Cbz-(NO_2)-Arg in 15 ml of anhydrous THF under nitrogen was added 1.0 g of 1,1'-carbonyl-diimidazole (Sigma). The solution was stirred at 10° C until all the solids dissolved and then cooled to -75° C (dry ice - acetone bath). To the solution was added 0.48 g of $LiAlH_4$ in 17 ml of THF over 30 min and the mixture stirred for 3 hr at -75° C under N_2. Unreacted $LiAlH_4$ was decomposed by the addition of 50 ml of cold 2 N HCl to pH 3.0. The reaction was extracted 3 times with $CHCl_3$, the $CHCl_3$ extract washed with H_2O, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under vacuum on a rotary evaporator. The white residue (impure Cbz-(NO_2) Argal) was dissolved in 80 ml of 70% ethanol to which 1.20 g of semi-carbazide·HCl and 1.84 g of sodium acetate was added. The solution was heated at 80° C for 15 min, and the solvent evaporated under vacuum. The residue was purified on a silica gel column eluted with 10% MeOH in $CHCl_3$, yielding 1.50 g (66.4% yield) of Cbz-(NO_2)-Arginal-SC, recrystallized from absolute EtOH, mp 105-108° C (uncorr.) (Lit. (9): 107-109° C), tlc over silica gel G plates gave a single spot of R_f = 0.19 in 90:15 $CHCl_3$:MeOH.

Synthesis of Argininal (Argal) Resin. To 200 mg of Cbz-(NO_2)-Argal-SC in a solution of 20 ml MeOH, 2 ml acetic acid and 1 ml H_2O, with 25 mg of palladium black was added 20 lb/in^2 pressure of H_2 in a Parr Hydrogenator, and the mixture shaken overnight. The mixture was degassed, the palladium centrifuged down, the residue washed 2 times with MeOH, the wash combined with original supernatant solution and the combined solvents evaporated under vacuum, yielding Argal-SC diacetate, tlc over silica gel plate gave single spot near origin in 90:15 $CHCl_3$:MeOH that gave positive ninhydrin and Sakaguchi (10) reagent reactions. The Argal-SC was dissolved in 4:1 MeOH:phosphate buffer(0.025 M sodium phosphate, pH 7.5), the solution cooled to 4° C, and 12 ml of Affi-Gel 10 (Bio-Rad Corp.) added. The mixture was shaken overnight at 4° C, the resin washed with MeOH:H_2O (4:1) to remove excess Argal-SC, the resin added to 10 ml of 1 M ethanolamine at pH 8.0, stirred for 2 hr, the resin collected and washed with H_2O. To remove the semicarbazone protecting group the resin was added to a solution of MeOH:acetic acid:formaldehyde (5:1:1) and mixed overnight at 23° C. The Argal resin was collected on a funnel, washed with H_2O, and stored in H_2O at 4° C prior to use.

Synthesis of Model Ethanolamine Resin. To 10 ml of Affi-Gel 10 was added 10 ml of 1 M ethanolamine at pH 8.0, the solution mixed for 3 hr at room temperature, the resin collected on a funnel and washed with cold H_2O. The resin was stored in H_2O at 4° C.

Chemical analyses for guanidino function in argininal-resin was carried out utilizing Sakaguchi reagent as modified by Messineo (10).

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[1]Abbreviations: Cbz, carbobenzoxy; Argal, Argininal; SC, semicarbazone; Bz, benzoyl; EE, ethyl ester; tlc, thin layer chromatography; MeOH, methanol; EtOH, ethanol; THF, tetrahydrofuran; NaOAc, sodium acetate; HOAc, acetic acid; Affigel, Affi-Gel 10 (Bio-Rad Corp).

Quantitative analysis for the aldehyde concentration in the Argal-resin was based on an analytical procedure previously described by Ulbrich and Schellenberger (11), as follows. To 0.1 ml of Argal-resin is added a saturated solution of p-phenylazoaniline in EtOH and acetic acid (ratio 10:1), the mixture incubated for 1 hr, and the resin washed with EtOH until the eluent is colorless. The resin is added to 1 ml of 8:1:1 MeOH:acetic acid:salicylaldehyde and shaken for 30 min, the supernatant collected, and the procedure repeated 2 more times with the same solvent. The supernatants are combined, made up to 5.0 ml, and the absorbance read at 405 nm with respect to a solution obtained by treatment of model ethanolamine-resin in the same way. The extinction coefficient for the Schiff base adduct of p-phenylazoanaline and salicylaldehyde was determined on a sample of this product independently synthesized by procedures previously described (11).[2]

α-Chymotrypsin (Worthington Bioch., 3X recry.) and trypsin (Sigma, 2X recry.) were assayed with BzTyrEE (12) and TosylArgEE (12), respectively.

Results Section.

Synthesis of Argal-Resin. Argal-SC is first synthesized by reducing the carbonyldimidazole ester of Cbz-(NO_2)-Arg with $LiAlH_4$ to its aldehyde form, Cbz-(NO_2)-Argal, protection of the aldehyde function as a semicarbazone (Cbz-(NO_2)-Argal-SC), and purification by silica gel chromatography. The Cbz and ω-NO_2 protecting groups of Cbz-(NO_2)-Argal-SC are removed by catalytic hydrogenation over Pd. The affinity resin is then generated by reacting Argal-SC with a crosslinked agarose beaded gel containing a succinylated diaminoethyl spacer group (10 atoms) and activated on its COOH end by an N-hydroxysuccinimide ester (Affi-Gel 10, Bio-Rad Labs.Inc.). The semicarbazone protecting group of the Arg-SC ligand is removed by treating the resin with MeOH:acetic acid:formaldehyde (37%) overnight (Fig. 1) In this reaction the semicarbazide is transfered from Argal to formaldehyde in a reaction catalyzed by acetic acid. The formation of aldehyde in this reaction is qualitatively followed by reaction with 2,4-dinitrophenyl hydrazine, which gives a yellow color. Alternatively, quantitative analysis of aldehyde concentration is carried out by reaction of the Arginal-resin with excess p-phenylazoaniline, which forms a Schiff base with the aldehyde groups. After washing the excess p-phenylazoaniline from the resin, the modified resin is treated with salicylaldehyde resulting in the transfer of the p-phenylazoaniline from Argal-resin to salicylaldehyde. This adduct

[2]Synthesis carried out by Dr. P.N.A. Murty according to procedures in reference 11.

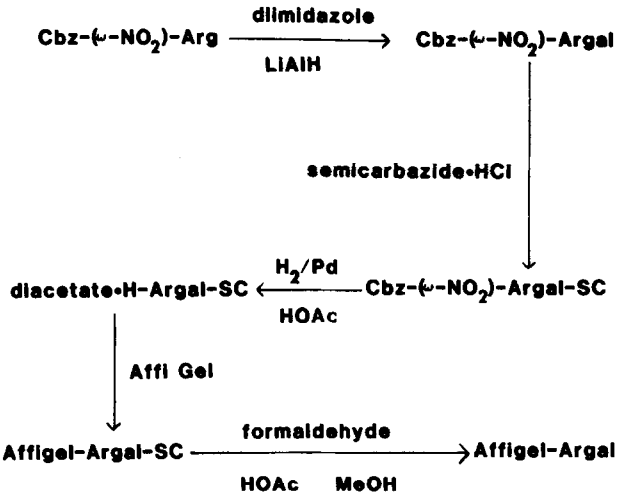

Figure 1. Synthetic Scheme Utilized in the Synthesis of Argininal Liganded Affinity Resin.

(Fig. 2) is eluted from the resin and quantitated by its absorbance at 405 nm. A comparison of the absorption obtained from the p-phenylazoaniline-salicylaldehyde adduct from the Argal-resin compared with an ethanolamine-resin blank showed a minimum of 0.1μMole/ml of aldehyde in the Argal-resin.

Association of Trypsin and Chymotrypsin to the Argal-Resin and Method of Elution. In a typical experiment, to 1 ml of Argal-resin in 0.025 M sodium phosphate (pH 6.0) in a small column (i.d. 7mm) was added a mixture of 0.5 mg α-chymotrypsin and 0.5 mg trypsin. The resin was washed with pH 6.0 buffer eluting in the first 10 ml 100% of the chymotrypsin activity placed on the column. The trypsin activity remained bound to the resin even after exhaustive washing. The elution of trypsin activity was not possible even with 2 N NaCl at pH values between 2.0 and 6.0. However, essentially all the trypsin activity was eluted from the Argal-resin on incubation of the resin with 0.1 N semicarbazide and 0.1 N sodium acetate (pH 3.8) for 15-30 min, and then eluting the trypsin in 10 ml of the same reagent.

Control experiments were carried out utilizing Arg-SC and ethanolamine-resin. To the later resin no affinity of either chymotrypsin or trypsin was observed in pH 6.0 (0.025 M sodium phosphate) buffer. The trypsin associated

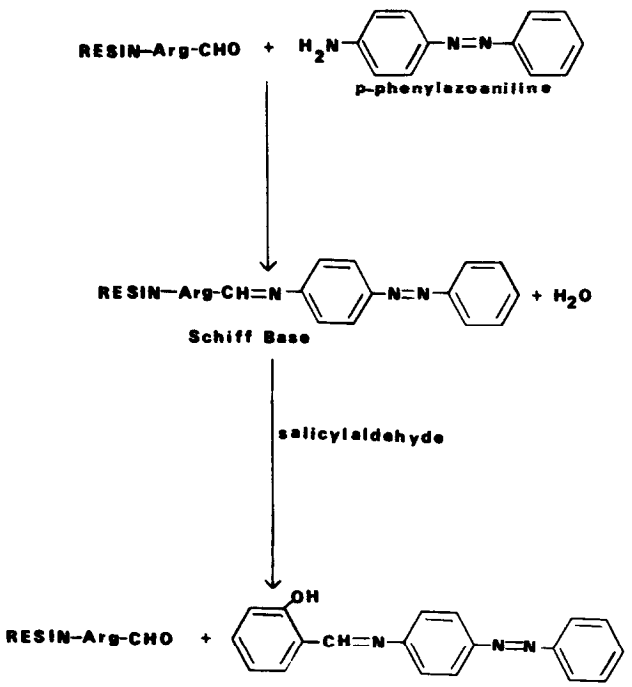

Figure 2. Scheme Utilized to Analyze Argininal-Resin for Aldehyde Concentration.

with the Arg-SC-resin at pH 6.0 (0.025 M sodium phosphate) buffer, but was eluted in 1 N NaCl phosphate buffer at pH 6.0.

Regeneration of the active Argal-resin after semicarbazide treatment to elute the trypsin was accomplished by treatment with MeOH:acetic acid:form aldehyde(37%) overnight, as previously described in the prodedure for synthesis of the Argal-resin.

Discussion Section.

The affinity of the Argal-resin is significantly greater than to affinity resins in which trypsin in noncovalently associated. This is shown by the ability to elute trypsin from the noncovalent affinity resins by high salt and/orlow pH, whereas the Argal-resin binds trypsin irreversibly under these conditions and trypsin is only eluted after addition of semicarbazide to the eluting buffer. This high affinity is due to the formation of a hemiacetal bond between the active site serine of trypsin and the aldehydic function of the Argininal, with a transition-state like geometry (1-3). The preferential affinity to trypsin over

chymotrypsin clearly shows the guanidinium group is required for selectivity and specificity of binding.

The Argal-resin serves as a model for the preparation of highly effective affinity resins using specific peptidyl aldehyde ligands. The tighter binding to the Argal ligand that to noncovalent binding resins show that the Argal resin can be used for the affinity purification or inhibition of proteases at significantly lower concentration than noncovalent type affinity resins. In addition, as peptidyl aldehydes appear to associate tightly to proteases independent of pH between pH 3 and 8 (1,4), affinity purification of proteases can be carried out at pH < 7 at which pH values many important protease are catalytically inactive and, thus, autocatalytic degradation will not be of consequence.

The Argal group may be added to peptidyl ligands of particular sequences with a high specificity towards a particular protease thus separating for example a particular coagulation protease from other coagulation proteases for which the peptidyl ligand has no special affinity. The transition-state like properties of peptidal aldehyde complex with enzyme will give an amplification of the specificity differences normally shown by the respective peptides without the Argal function .

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