Communications to the Editor

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CONVENIENT PURIFICATION OF HUMAN SPLEEN FIBRINOLYTIC PROTEINASE (SFP) AND HUMAN LEUCOCYTE ELASTASE BY AFFINITY CHROMATOGRAPHY[1)]

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A peptide inhibitor, Suc-L-Tyr-D-Leu-D-Val-pNA, for human spleen fibrinolytic proteinase (SFP) and for human leucocyte elastase was coupled with AH Sepharose 4B using water soluble carbodiimide. A 116-fold purification of the crude extract of SFP and a 59-fold purification of the partially purified leucocyte elastase were achieved by (Suc-L-Tyr-D-Leu-D-Val-pNA)-AH Sepharose 4B affinity chromatography.

KEYWORDS—peptide inhibitor; Suc-L-Tyr-D-Leu-D-Val-pNA; human spleen fibrinolytic proteinase (SFP); human leucocyte elastase; (Suc-L-Tyr-D-Leu-D-Val-pNA)-AH Sepharose 4B; affinity chromatography; purification of enzyme

Studies are under way in our laboratories directed to the synthesis of substrates and inhibitors in order to investigate the enzymes, human spleen fibrinolytic proteinase (SFP) and human leucocyte elastase. Okamoto et al.,[2)] described the isolation and the purification of a neutral proteinase which degrades fibrin and fibrinogen from the human spleen tissue and they reported on some properties of the enzyme. Most of its properties, such as molecular weight and mobility in electophoresis, are similar to those of the human leucocyte elastase; however, SFP does not degrade elastin. Recently, it was found that the newly synthesized chromogenic substrate Suc-L-Tyr-L-Leu-L-Val-pNA, specific for SFP,[3,4)] is also a good substrate for human leucocyte elastase.[5)] Also Suc-L-Ala-L-Tyr-L-Leu-L-Val-pNA is a much more favorable substrate for SFP and leucocyte elastase.[5)] We also reported that some of the stereoisomers of Suc-Tyr-Leu-Val-pNA inhibit the amidolytic activity of SFP and that of those stereoisomers, Suc-L-Tyr-D-Leu-D-Val-pNA is the most effective inhibitor of SFP.[6)] The elastase activity is also inhibited by those stereoisomers in a manner similar to SFP (unpublished data). During these investigations, a simple and improved purification procedure for SFP and elastase was required in order to minimize the purification steps and obtain higher yields of enzymatic activity for further studies. Affinity chromatography is a very convenient method to purify enzymes.[7)] It was our idea that the synthesis of affinity Sepharose using a synthetic peptide inhibitor specific for SFP and leucocyte elastase would make possible the simple purification of those enzymes.

This communication deals with the synthsis of affinity Sepharose as well as the simple purification procedure for SFP and the human leucocyte elastase.

First, we attempted to synthesize more effective inhibitors for SFP and the elastase. Since Suc-L-Ala-L-Tyr-L-Leu-L-Val-pNA was found to be a more favorable substrate for SFP and the elastase [k_{cat}/K_m values ($M^{-1}s^{-1}$); 84,000 and 22,300 respectively], four kinds of stereoisomers having the D-Leu-D-Val-pNA moiety at the C-terminus were synthesized; Suc-L-Ala-L-Tyr-D-Leu-D-Val-pNA [mp 243-247°C, $[\alpha]_D^{25}$ +60.2° (c=1.0, MeOH), Anal. Calcd for $C_{33}H_{44}N_6O_{10}$: C, 57.88; H, 6.47; N, 12.27. Found: C, 57.90; H, 6.66; N, 11.97], Suc-L-Ala-D-Tyr-D-Leu-D-Val-pNA [mp 221-226°C, $[\alpha]_D^{25}$ -67.3° (c=1.0, MeOH), Anal. Calcd for $C_{33}H_{44}N_6O_{10}$: C, 57.88; H, 6.47 ; N, 12.27. Found: C, 58.15; H, 6.76; N, 12.02], Suc-D-Ala-L-Tyr-D-Leu-D-Val-pNA [mp 229-233°C, $[\alpha]_D^{25}$ +71.2° (c=1.0, MeOH), Anal. Calcd for $C_{33}H_{44}N_6O_{10}\cdot H_2O$: C, 56.40; H, 6.59; N, 11.95. Found: C, 56.41; H, 6.63; N, 11.38] and Suc-D-Ala-D-Tyr-D-Leu-D-Val-pNA [mp 228-230°C, $[\alpha]_D^{25}$ +30.7° (c=1.0, MeOH), Anal. Calcd for $C_{33}H_{44}N_6O_{10}$: C, 57.88; H, 6.47; N, 12.27. Found: C 57.45; H, 6.51; N, 12.03]. Although these stereoisomers inhibited SFP and the elastase activities, their effectiveness was comparable to that of Suc-L-Tyr-D-Leu-D-Val-pNA. So we prepared affinity Sepharose using Suc-L-Tyr-D-Leu-D-Val-pNA and AH Sepharose 4B as follows. Suc-L-Tyr-D-Leu-D-Val-pNA (100 mg, 160 μmol) and AH Sepharose 4B (Pharmacia Fine Chemicals, 5 g, amino group content; 24-40 μmol/g) were coupled using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride in dioxane-H_2O (1:1, 40 ml) at room temperature overnight. After washing with the same solvent, affinity Sepharose was further washed with water. The content of inhibitor was calculated from amino acid analysis of an acid hydrolysate (6 N HCl, 110°C, 20 h) of Suc-L-Tyr-D-Leu-D-Val-pNA-Sepharose (38 μmol/g Sepharose).

In order to purify SFP and the leucocyte elastase, the obtained Sepharose was diluted 50 times with Sepharose 4B and a column (2 x 5 cm) was prepared and equilibrated with Tris-HCl buffer (0.1 M, pH 8.0) containing 0.5 M $NaClO_4$. The purification procedure of SFP was as follows. The spleen tissue was thawed at room temperature, homogenized with 10 volumes of physiologic saline and centrifuged at 100,000 g for 100 min at 4°C. The precipitate obtained was weighed and re-homogenized in 10 volumes of Tris-HCl buffer (0.1 M, pH 8.0) containing 2M $NaClO_4$. After extraction at 4°C for 120 min, the homogenate was centrifuged at 100,000 g for 100 min. The supernate was used as the crude spleen extract. The extract was diluted 4 times with Tris-HCl buffer (0.1 M, pH 8.0) and adsorbed on the affinity column. After the most inactive protein was eluted, the column was washed with Tris-HCl buffer (0.1 M, pH 8.0) containing 2 M $NaClO_4$. The elution of the desired enzyme (SFP) was achieved with Tris-HCl buffer (0.1 M, pH 8.0) containing 8 M urea. In order to detect the location of SFP, the synthetic substrate, Suc-L-Ala-L-Tyr-L-Leu-L-Val-pNA was employed. The specific activity and purification factor of SFP before and after affinity chromatography are summarized in Table I.

Table I. Specific Activity and Purification Factor of SFP

	Total activity(U)[a]	Total protein(mg)	Specific activity(U/mg)	Purification factor
Crude Extract	800	560	1.43	1
Affinity Chromatography	390	2.35	166	116

a) The release of 1 μmol pNA from 0.3 mM Suc-L-Ala-L-Tyr-L-Leu-L-Val-pNA by 1 ml of enzyme for 60 min was defined as 1 unit of enzyme activity.

It can be seen from the Table I that a 116-fold purification of SFP was achieved by the rapid one step purification on the affinity gel and that recovery of enzymatic activity was about 50%.

The purification of human leucocyte elastase was performed in essentially the same way described above using partially purified human leucocyte elastase as the starting material. A 59-fold purification of the elastase was achieved on this affinity gel and the recovery of enzymatic activity was 85%.

It was found that other proteins and enzymes such as chymotrypsin, trypsin, porcine pancreatic elastase, plasmin, thrombin and urokinase were not bound with the affinity gel. This column was fairly durable and the selective ability was not reduced after repeated use (at least 5 times, peptide content: 95% of original affinity Sepharose) at room temperature. From these investigations, it was concluded that this affinity column is a useful tool to purify not only SFP but also human leucocyte elastase which has attracted our attention because of its involvement in tissue destruction in pulmonary emphysema.[8)]

REFERENCES AND NOTES

1) Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: Biochemistry, 5, 2485 (1966); ibid., 6, 362 (1967); ibid., 11, 1726 (1972). Suc=succinyl, pNA=p-nitroanilide.
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