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Characterization of Sepharose-Bound Pronase**

by

Si Myung Byun and Finn Wold*

Department of Biological Science and Technology, Korea Advanced Institute of Science Seoul, Korea
Department of Biochemistry, University of Minnesota St. Paul, Minnesota 55108 *U.S.A.**

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固定化 Pronase의 特性에 관한 硏究**

변시명 · 배은옥*

한국과학원 생물공학과 · 미국 미네소타 주립대학교 생화학과*

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SUMMARY

Two kinds of sepharose-bound pronases were successfully prepared. The immobilized pronase, directly coupled to cyanogen-bromide activated sepharose, retains 22.6% of original specific activity against casein. However, ω-aminoalkyl sepharose immobilized pronases, in which extension arms of ω-aminoalkyl group (number of $-CH_2-$ is 8, 10, and 12) are used, retain almost 100% of original specific activity. Studies of enzyme stability, pH dependence, temperature dependence, and Km values are presented.

INTRODUCTION

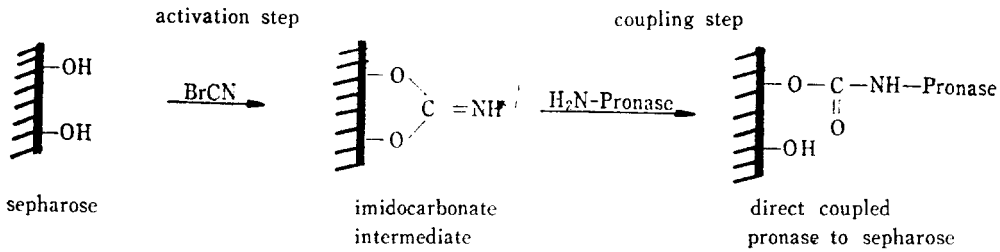
The utilization of enzymes as catalysts has been limited mainly because costs of enzyme are sometimes too expensive. However, development of immobilization techniques for enzymes, has made the repeated use possible. Immobilization of enzymes, therfore, has been the subject of increased interest and their significance and applicability have reviewed[1,2] in the areas of theraputics, syntheses, analyses and food processings[3,4,5]. The fact that many of soluble enzymes are not on the GRAS list[6] further hampered the use of soluble enzyme in food processing. In these regards, together with their easy removal from reaction mixtures, immobilized proteases have been investigated for use in chill proofing beer[7], continuous coagulation of milk[8], and inhibition of oxidative rancidity in milk[9].

Pronase produced by *Streptomyces griseus* has very broad specificity of proteolytic activity[10,11], which will digest virtually any protein to free amino acids. Therefore, pronase has been coupled to various solid matrix[12,13]. However, pronounced substrate exclusion effects are exhibited especially for large substrate molecules such as casein or BSA.

This report includes a description of a new method of ω-aminoalkyl sepharose immobilized pronase which may release the steric restriction imposed on the

** This research was supported financially by Department of Biochemistry, University of Minnesota, St. Paul, Minn. Abbreviations used in this report are as follows : GRAS, generally recognized as safe; BSA, bovine serum albumin; PUK, proteinase activity unit of Kakein; LPNA, leucine-p-nitroaniline.

1. Direct Coupling of Pronase;



2. Coupling of α,ω-diaminoalkane and Pronase:

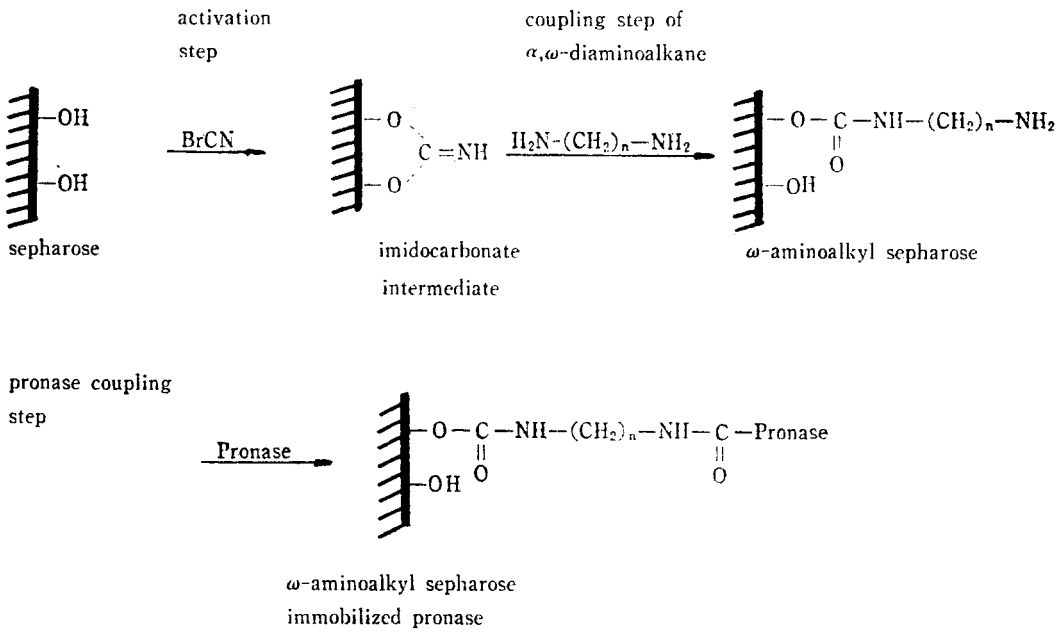


Fig. 1: Schematic Diagrams of Pronase Coupling to Sepharose

matrix by giving increased flexibility and mobility of the expansion arm, and its characteristics. Two preparation methods are shown in Fig. 1.

MATERIALS AND METHODS

Commercial pronase and Tris were obtained from Cal. Biochem. Sepharose 4B was supplied by Pharmacia Fine Chemicals. α,ω-$H_2N(CH_2)n$-NH_2(n=2, 3, 4, 6, 10 and 12), 1-cyclohexyl-3(2-morpholinoethyl)-carbodiimide-metho-p-toluenesulfonate were products of Aldrich Chem. LPNA, BSA, and p-nitroaniline were purchased from Sigma.

Enzyme Assays : The extent of casein or BSA digestion was determined by measuring the optical density (660 nm) of the TCA soluble peptides with tyrosine as standard[14]. Activity was expressed with PUK (proteinase activity unit of Kakein). One unit of PUK has been defined as activity of enzyme which liberates, per min. at 40°C, a digestion product equivalent to 25μg of tyrosine. Hydrolysis of LPNA was followed spectrophotometrically by monitoring p-nitroaniline at 405 nm[15]. Since activity of immobilized pronase depends on the shaking during the incubation, appropriate aliquot of immobilized enzyme suspension was incubated in the constant temperature bath equi-

pped with water-immersed magnetic stirrer under the same incubation method described above. In this way immobilized enzyme was constantly stirred and activity assay was reproducible.

Activation of Sepharose 4B : Sepharose 4B was activated with cyanogen bromide at room temperature in a well-ventillated hood according to the procedure of March *et. al.*[16]. Ten ml of washed Sepharose 4B, consisting of equal volumes of gel and water, is added to 10 ml of 2M sodium carbonate and mixed slowly. A solution of 0.5 g of cyanogen bromide in 0.25 ml of acetonitrile is added all at once. The slurry is stirred vigorously for 2 min., after which the slurry is poured onto a coarse sintered-glass funnel, washed with 100 ml each of cold 0.1M sodium bicarbonate, pH 9.5, cold water, and cold 0.1M sodium borate buffer, pH 7.5 containing 0.05M HCl, and 50mM $CaCl_2$.

Direct Coupling of Pronase to Activated Sepharose : To 10 ml of activated Sepharose 4B slurry, 20 ml of enzyme solution (50mg in the buffer) was added and gently swirled overnight at cold room. The beads were collected on a coarse glass filter and washed with 300 ml each of cold water, 0.5M NaCl, 2M urea and finally 0.1M sodium borate buffer, pH 7.5 containing 0.05M HCl and 50mM $CaCl_2$. Immobilized pronase was resuspended in 20 ml of 0.03M HCl and stored at refrigerator.

Coupling of α,ω-diaminoalkane and Pronase to Activated Sepharose : Ten ml of activated Sepharose 4B suspended in cold 0.1M sodium bicarbonate, pH 9.0 was mixed with 10 ml water containing 10 mmoles of α,ω-diaminoalkane [$NH_2(CH_2)nNH_2$, n=2, 3, 4, 6, 10 and 12. For n=3, hydroxyl group at 2 position, i.e, 1,3-diamino-2-hydroxyl propane was used]. The pH of the diamine solution was adjusted to 9.0 with 6N-HCl before use. Coupling was performed at 4°C overnight as described above. After reaction was completed the ω-aminoalkyl sepharose was washed with cold water, 0.1M $NaHCO_3$, pH 9.0, 0.05M NaOH, water, 0.1M CH_3COOH and water. Finally bead was suspended in 10 ml of 0.1M sodium borate buffer containing HCl and $CaCl_2$. Coupling of α,ω-diaminoalkane was tested qualitatively by 2,4,6-trinitro-benzenesulfonate method (TNBS)[17] for a small aliquot of beads. All preparations were color positive for this test. This ω-aminoalkyl sepharose suspension was mixed with 10ml of the buffer solution containing 40 mg of 1-cyclohexyl -3(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate and 50mg of pronase. After 30 min. pH of the mixture was adjusted to pH 7.0 and reaction continued overnight at cold room. The sepharose gel was filtered and washed with cold water, 0.5M NaCl, 2M urea thoroughly. Finally immobilized pronase was suspended in 20 ml of 0.03M HCl and stored at 4°C.

Determination of Protein Bound to Sepharose. Immobilized enzyme was filtered on a glass filter and washed with distilled water thouroughly. Wet caked bead was weighed out and hydrolyzed with 2 ml of 6N HCl in a sealed tube at 110°C oven for 24 hrs. After hydrolysis, HCl was removed by vacuum and the residue was dissolved in 0.1M sodium citrate buffer, pH 4.5 and filtered. Amino acid composition was dertermined with a fully automated Beckman model 119 amino acid analyzer. With a comparison of soluble pronase, pretein bound was calculated on the dry base of the gel bead.

Kinetic Studies : Soluble and immobilized pronases were characterized in terms of pH, temperature and substrate concentration. Both casein (or BSA for pH dependence) and LPNA were used as substrates.

Since the enzyme activities were directly proportional to the incubation time up to 30 min for casein or BSA, and 5 min for LPNA, relative activities at 5 min for casein or BSA and 3 min for LPNA were used instead of initial velocities. The pH dependence of the activity was determined as same procedure described under enzyme activity assay except changing the pH of incubation mixture. 0.05 M citrate-phosphate buffers between pH 3.0 to 7.0, 0.05 M Tris HCl buffers between pH 8.0 to 9.0 and 0.05 M carbonate-bicarbonate buffers between pH 10 to 11 were used. All buffers used contain $CaCl_2$, 5 mM as final concentration. The effect of temperature on the enzyme activity was examined at 8 different temperatures in the range from 20°C to 80°C. Lineweaver-Burk plots were carried out with enzyme activities at 6 different LPNA concentrations at the range from 3.75×10^{-4} M to 3.0×10^{-3} M.

RESULTS AND DISCUSSION

The resulting pronases immobilized on various solid matrices are active against both large (casein and BSA) and small substrates (LPNA). The enzyme may be reused many times without loss of activity as it is kept moist. Table 1 shows the comparative results of various immobilized pronases. As comparison, soluble pronase is also included.

Table 1 : Comparative Results of Various Immobilized Pronases on Sepharose 4B

Enzyme	Total volume(ml)	Activity (PUK/ml. gel)	mg. Dry gel	mg. Protein	Specific activity (PUK/mg protein)
			mg. gel	g dry gel	
Soluble	(0.1mg/ml)	(6.95/ml)	—	—	69.5
Sepharose immobilized	20	5.6	7.4	42.8	15.7
ω-aminoalkyl sepharose-immobilized					
n=2	20	0	6.4	0	0
n=3	20	0	6.0	0	0
n=4	20	1.45	6.9	4.74	44.3
n=6	20	9.85	7.6	16.72	77.5
n=10	20	12.45	7.6	21.02	77.9
n=12	20	13.30	7.2	29.44	62.8

ω-aminoalkyl sepharose=Sepharose−NH−(CH_2)n−NH−

No pronase was bound to ω-aminoalkyl sepharose matrix in which the number of methylene groups in the extension arm is 2 and 3. No enzyme activities were observed though α, ω-diaminoalkane binding was observed by TNBS test. This may be due to the different mode of accessability of the protein when it is coupled.

It was reported that pronase exhibited prounced substrate exclusion effect when different carrier was used. As examples pronase coupled to a copolymer of leucine and p-aminophenylamine retained 20% of the native specific activity[12], and a glass derivative of pronase possessed 57% of the original specific activity against BSA[13]. Our results show 22.6% of original specific activity was retained for direct coupled pronase to sepharose and almost 100% original activity was retained for ω-aminoalkyl sepharose immobilized pronase in which the number of methylene groups in the extension arm is 6, 10 and 12. (Table 1). Since other methods immobilized the enzyme directly to the solid matrix, this may give a limited space for the enzyme to bind substrate and extension arm of ω-aminoalkyl group may allow it to contact easily large substrate molecule such as casein or BSA. In fact the dramatic effects of increasing the extension arm length may be the relief of steric restriction imposed by the matrix and in part the increased flexibility and mobility of the ligand as it protrudes further into the solvent. It is suggested that the use of an extension arm at least 57 Å long enables the nucleotide to traverse a barrier imposed by the micro-environment of the hydrophylic polymer[18]. This may be due to an ordered layer of water molecules surrounding the matrix backbone which restricts diffusion in this region, or to the vibrational motion of the lattice.

Figure 2 shows the retention of proteolytic activities of immobilized pronases at 4°C in the acidic conditions. Soluble pronase was also compared. Both directly immobilized pronase and ω-aminoalkyl sepharose pronase were stable in the moist form for at least 3 months. Stability of ω-aminoalkyl sepharose pronase may be due to intra- or inter-crosslinking of pronase itself. This possibility was tested with pronase and the carbodiimide compound as same coupling method except without sepharose gel. The product was not stabilized during storage in the same condition. After 2 weeks over 80% of original activity was lost.

The pH optima of the soluble and ω-aminoalkyl sepharose immobilized pronase are similar on both BSA and LPNA. However, direct sepharose immobilized

pronase was considerably broadened at the pH optima on both BSA and LPNA. (Figure 3 and 4)

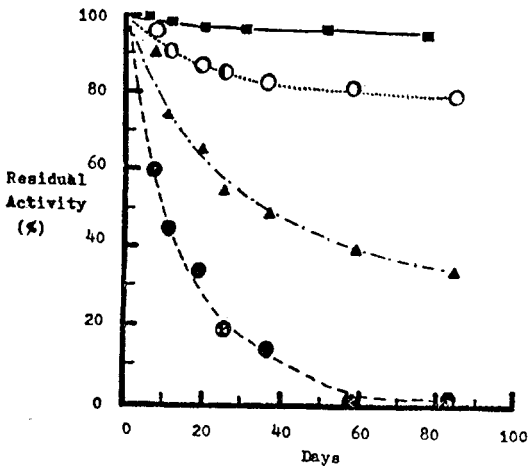


Fig. 2. Residual Caseinolytic Activities of Soluble and Immobilized Pronases During Storage in Acidic and Neutral Conditions at 4°C

●-·-●-·-● : soluble pronase in 0.03 M HCl, ▲—▲—▲ : soluble pronase in 0.1 M sodium borate buffer, pH 7.5 containing 0.05 M HCl and 50mM $CaCl_2$, ○···○···○ : direct coupled pronase to sepharose in 0.03 M HCl, ■—■—■ : ω-aminoalkyl sepharose immobilized pronase in 0.03 M HCl (n=6, 10, and 12).

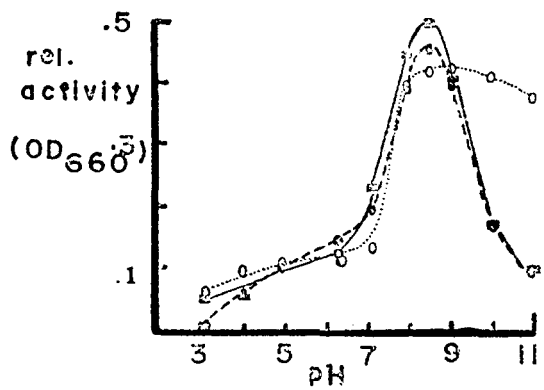


Fig. 3. pH Profiles of Soluble and Immobilized Pronases on BSA.

●-----●-----● : soluble pronase, ○···○···○: direct coupled pronase to sepharose, and ■—■—■ : ω-aminoalkyl sepharose immobilized pronase. (n=6, 10, and 12)

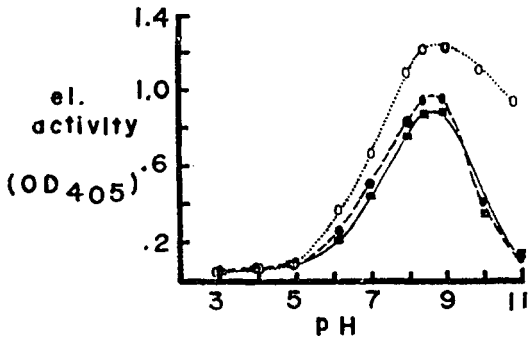


Fig. 4. pH Profiles of Soluble and Immobilized Pronase on LPNA.

●---●---● : soluble pronase, ○···○···○ : direct coupled pronase to sepharose, and ■—■—■ : ω-aminoalkyl sepharose immobilized pronase. (n=6, 10, and 12)

The effect of temperature on the hydrolyses of casein and LPNA by immobilized pronases was compared with that of soluble pronase and the results indicate that the optimal temperature for the immobilized pronase was 5 degree higher than that for soluble enzyme on casein as a substrate. For the estimation of the activation energies of soluble and immobilized pronases, Arrhenius plotting has completed as shown in Figure 5 and Figure 6. The activation energies calculated from Figugre 5 are 14.0 kcal/mole/deg for soluble pronase and 11.0 kcal/mole/deg for both immobilized pronases with casein as substrate. From Figure 6 the activation energies on LPNA are 9.7 kcal/mole/deg for soluble pronase, 11.6 kcal/mole/deg for direct coupled pronase to sepharose and 12.6 kcal/mole/deg for ω-aminoalkyl sepharose immobilized pronase. It is little ambiguous that the activation energy of the soluble enzyme (14.0 kcal) is higher than those of the immobilized (11.0 kcal) on the substrate casein.

Figure 7 shows Km values of various pronase preparations for LPNA as substrate when Lineweaver-Burk plots were analyzed. From this analysis Km's for LPNA appear 0.83 mM for soluble, 2.22 mM for direct coupled pronase to sepharose, and 0.91 mM for ω-aminoalkyl sepharose immobilized pronase.

Table 2 summarized these results.

Since soluble pronase is not on the GRAS list at the present time for food use, developing a system

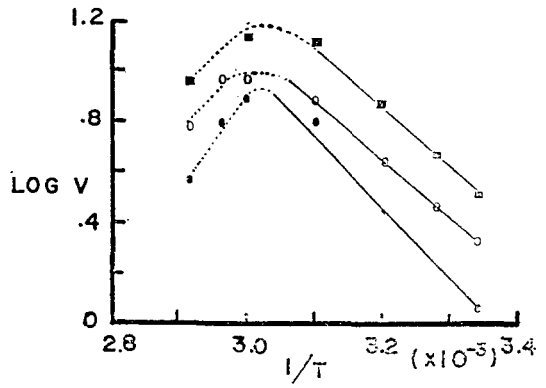


Fig. 5. Arrhenius Plots for Soluble and Immobilized Pronase Reaction on Casein.
●—●—●: soluble pronase ○—○—○: direct coupled pronase to sepharose, ■—■—■ : ω-aminoalkyl sepharose immobilized pronase (n=6, 10, and 12)

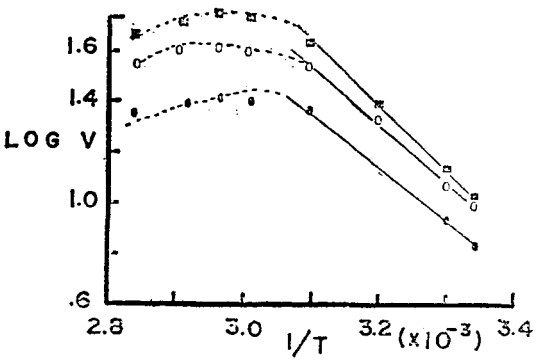


Fig. 6. Arrhenius Plots for Soluble and Immobilized Pronase Reactionon LPNA.
●—●—●: soluble pronase. ○—○—○: direct coupled pronase to sepharose, ■—■—■ : ω-aminoalkyl sepharose immobilized pronase (n=6, 10, and 12)

for protein hydrolysis using immobilized pronase offerse food producers several major advantages: continuous process rather than batch operation, lower cost

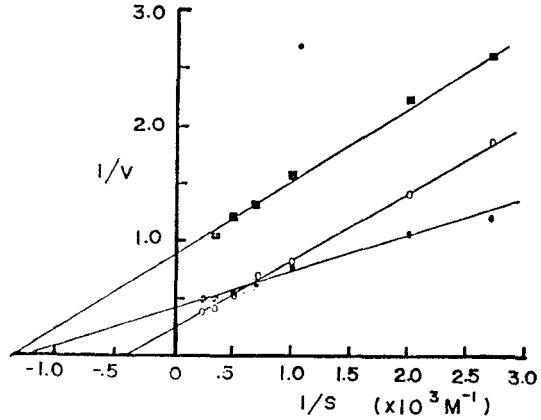


Fig. 7. Lineweavr-Burk Plots for Hydrolysis of LPNA by Soluble and Immobilized Pronase.
●—●—●: soluble pronase, ○—○—○: direct coupled pronase to sepharose ■—■—■ : ω-aminoalkyl sepharose immobilizd pronase (n=6, 10, and 12)

since the enzyme are reused, better control over the hydrolytic process with a use of other peptidase either simultaneously or sequentially, and probable permission of non-GRAS pronase.

Proteins and protein hydrolysate have application in many areas in the food industry. Protein extracts are presently used in beverages, baby food products and have potential as rich sources of L-amino acids. Generally proteins are hydrolyzed either to increase solubility, affect taste or improve digestability. The hydrolysis of these materials to produce these products can be carried out efficiently with immobilized pronase with combination of other immobilized peptidase.

Studies are now under way to determine the extent of soybean protein hydroysis, and chill proofing effects of beer by a mixture of immobilized pronase and immobilized carboxypeptidase.

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Table 2 : Kinetic Results of Soluble and Immobilized Pronase

Enzyme	pH Optima		Ea		Km
	BSA	LPNA	Casein	LPNA	LPNA
Soluble	8.5	9.0	14.0 kcal /mole.deg.	9.7 kcal /mole.deg.	0.83 mM
Sepharose immobilized	8.5—9.0	9.0	11.0	11.6	2.22
ω-aminoalkyl sepharose immobilized	8.5	9.0	11.0	12.6	0.91

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요 약

단백질 분해효소로서 넓은 특이성을 가지고 있는 Pronase를 사용하여 두 가지의 다른 방법으로 固定化酵素를 제조하였다. Sepharose에 직접 결합시킨 효소는 원래 효소활성도의 22.6%을 유지하고 있었지만, Extension arm으로서 $-CH_2-$ 기의 수가 6, 10, 12를 가진 ω-amino-alkyl group을 사용하여 Sepharose에 결합시킨 효소는 원래 효소활성도의 거의 100%를 다 유지하고 있었다.

제조한 固定化酵素의 pH 효과, 온도효과 및 Km을 조사하였으며 GRAS list에 들지 않은 Pronase를 식품가공에 이용하는 가능성을 고찰하였다.

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