Selective Separation of Trypsin from Pancreatin Using Bioaffinity in Reverse Micellar System Composed of a Nonionic Surfactant

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Received 10 June 1997; accepted 11 November 1997

Abstract: Selective separation of trypsin from a mixture involving many kinds of contaminating proteins, i.e., pancreatin, was achieved using trypsin inhibitor immobilized in the reverse micelles, which were composed of a nonionic surfactant, tetra-oxyethylene monodecylether. To determine the efficient operations throughout the whole separation process we examined the operating conditions, which affect the immobilization efficiency of trypsin inhibitor and also the forward and backward extractions of trypsin. Fifty percent of the recovery of trypsin from pancreatin was realized with no loss of activity of the recovered trypsin. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **58**: 649–653, 1998.
Keywords: bioaffinity separation; pancreatin; trypsin; reverse micelles; nonionic surfactant

INTRODUCTION

Selective separation of a target protein from a mixture containing many contaminating proteins is a central issue for bioaffinity separation. However, the selective separation has not been sufficiently achieved in reverse micellar systems, because the reverse micelles composed of ionic surfactants can extract many kinds of proteins through electrostatic interaction under usual operating conditions (Chen and Jen, 1994; Coughlin and Baclaski, 1990; Kelly et al., 1993a,b; Paradkar and Dordick, 1991, 1993; Woll et al., 1989).

We used a nonionic surfactant, tetra-oxyethylenemonodecylether (C10E4) instead of ionic surfactants such as Aerosol-OT. Water-soluble proteins in a bulk aqueous solution are difficult to be solubilized in the reverse micelles composed of nonionic surfactants alone in the two-phase extraction system, because strong interaction does not work between the micelles and the proteins. The driving force of the extraction is originated from the entropic effect, i.e., the dispersion of proteins to the water pools of the micelles (Shioi et al., 1997). In fact, the reverse micelles composed of C10E4 hardly extract water-soluble proteins such as cytochrome c, lysozyme, chymotrypsinogen, and concanavalin A (Adachi et al. 1996, 1997). Furthermore, nonionic surfactants do not cause severe denaturation of proteins due to lack of the electrostatic interaction. Thus, reverse micelles composed of nonionic surfactants provide an ideal system for selective separation of proteins if a protein-specific interaction is introduced into the system.

We reported that concanavalin A was extracted selectively from contaminating proteins by using alkyl-pyranoglucosides as affinity ligands (Adachi et al., 1996). We extended this method to a typical protein-protein interaction system, i.e., trypsin trypsin inhibitor (*TR*-*TRI*) system (Lei & Reeck, 1986; Steiner & Frattali, 1969). Trypsin from bovine pancreas (B-*TR*) was selectively separated by the forward and backward extractions using *TRI*, which was immobilized in the reverse micelles composed of nonionic surfactant by covalently combining cholesteryl groups (Adachi et al., 1997).

In this article, we examine the conditions for efficient immobilization of *TRI* and the subsequent extractions. Next, we present, from a more practical viewpoint, selective separation of porcine trypsin (P-*TR*) from many contaminating proteins, i.e., pancreatin from porcine pancreas, using the reverse micelles containing immobilized *TRI*.

MATERIALS AND METHODS

Materials

Pancreatin from porcine pancreas and trypsin from porcine pancreas (P-*TR*) type IV were purchased from Sigma Chemical Co. Tetra-oxyethylene-monodecylether (C10E4), trypsin inhibitor (*TRI*), trypsin from bovine pancreas (B-*TR*), Cholesteryl-chloroformate(CC), α *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and other materials such as *n*-hexane are the same as those described in a previous article (Adachi et al., 1997).

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 CCC 0006-3592/98/060649-05

Immobilization of Trypsin Inhibitor in Reverse Micelles

We can solubilize *TRI* into the reverse micelles composed of C10E4 by the injection method, although almost no *TRI* is solubilized by the phase-transfer method in the two-phase system as mentioned above. We investigated the effects of the operation parameters on the solubilization and immobilization efficiency, i.e., the W_0-value (water-to-surfactant mole ratio in the reverse micellar phase), pH, and the concentration of CC. A typical procedure is as follows: *TRI* (20 mg) was dissolved in 0.35 mL of 0.1*M* borate buffer (pH 12), and this *TRI* solution was mixed with 10 mL of 0.2*M* C10E4 solution of *n*-hexane to solubilize in the reverse micelles. Then, 1 mL of 0.1*M* cholesteryl-chloroformate in *n*-hexane was added to the reverse micellar solution of C10E4, and cholesteryl groups were introduced to the amino groups of the lysine residues of *TRI* for about 20 h at 25°C (Michel and Pileni, 1994). Finally, the reverse micellar phase was washed with 0.05*M* Tris-HCl buffer solution containing 0.2*M* NaCl (pH 8) to remove water-soluble components. The amount of immobilized *TRI* was determined from what remained in the reverse micellar phase after washing. At this stage, the W_0-value became 25—the equilibrium value in the reverse micelles/Tris-HCl buffer two-phase system.

Forward and Backward Extractions of Porcine Trypsin from Pancreatin

The extraction behavior of P-*TR* without contaminating proteins was investigated prior to the extraction of P-*TR* from pancreatin. The procedure was the same as that of B-TR described in a previous article (Adachi et al. 1997). The forward extraction procedure for P-*TR* from pancreatin was as follows. Pancreatin (100 mg) was dissolved in 10 mL of 0.05*M* Tris-HCl buffer (pH 8) containing 0.2*M* NaCl. Since the aqueous solution was turbid slightly, the solution was centrifuged, because P-*TR* dissolved in the solution completely. After centrifugation, the supernatant solution was contacted with the same volume of reverse micellar phase-containing immobilized *TRI* (the concentration ratio $[\overline{TRI}]_{Im}/[\text{P-}TR]_F$ was mostly 2), and P-*TR* was extracted. Before back-extraction, the reverse micellar phase was washed by 0.05*M* Tris-HCl (pH 8) buffer containing 0.2*M* NaCl. Backward extraction of P-*TR* from the reverse micellar phase was performed successfully using 0.015*M* hydrochloric acid aqueous solution containing 0.2*M* NaCl.

Measurements of Concentration and Activity of Porcine Trypsin

The concentrations of P-*TR* were measured from the optical density at 280 nm (the molecular absorption coefficient was 3200 m^2/mol) when it existed without contaminating proteins. The concentration of *TRI* and the activity of P-*TR* (Erlanger et al., 1961) were determined by the same methods as those in a previous article (Adachi et al., 1997). The concentration of P-*TR* in pancreatin was determined by hydrophobic-interaction liquid chromatography with a Shodex HICPH-815 column (length 7.5 cm, diameter 8 mm) at 0.8 mL/min by a linear gradient from 1.7*M* ammonium sulfate to 10 m*M* borate buffer solution (pH 7.5) for 30 min.

RESULTS AND DISCUSSION

Immobilization of Trypsin Inhibitor

The effect of W_0-value on the solubilized concentration of *TRI* in the reverse micellar phase $[\overline{TRI}]_S$ was examined by injecting the borate buffer solution containing various amounts of TRI to the reverse micellar phase. Hereafter, over bar represents the concentration in the reverse micellar phase. Figure 1 shows the relationship between $[\overline{TRI}]_S$ and $[TRI]_F$. Here, $[TRI]_F$ is defined as the feed amount of *TRI* divided by the total volume of the reverse micellar phase. When $W_0 = 5$, $[\overline{TRI}]_S$ is low and almost constant regardless of $[TRI]_F$. Whereas, when $W_0 = 10$, almost all *TRI* were solubilized in the reverse micellar phase. The diameter of the water-pool of the reverse micelle is less than that of *TRI* when $W_0 < 10$ (Shioi et al., 1997). Thus, *TRI* was rejected by these reverse micelles (Kawakami et al., 1996; Mat & Stuckey, 1993). When $W_0 > 12.5$, the excess aqueous phase appeared. Trypsin inhibitor was distributed to this deposited aqueous phase, resulting in the decrease in $[\overline{TRI}]_S$. Thus, the best W_0-condition for the *TRI*-solubilization is 10.

The effect of pH on the immobilization of *TRI* is shown in Figure 2. The immobilized fraction, i.e., the ratio of the immobilized concentration of *TRI*, $[\overline{TRI}]_{Im}$, to $[\overline{TRI}]_S$, increased with increasing pH-value, because acylation reaction produces hydrochloric acid. The effect of the feed concentration ratio RC ($= [\overline{CC}]_F/[TRI]_F$) on the immobiliza-

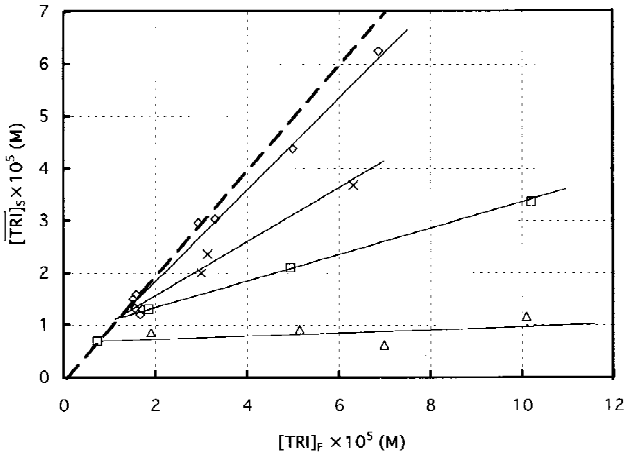

Figure 1. Effect of W_0 on solubilized concentration of *TRI* $[\overline{TRI}]_S$ in reverse micellar-phase composed of 0.2*M* C10E4/n-hexane. $[TRI]_F$ represents the feed amount of TRI divided by the toral volume of the reverse micellar phase. TRI contained in 0.1*M* borate buffer (pH 12) was injected to reverse micellar phase (△: $W_0 = 5$, □: $W_0 = 7.5$, ◇: $W_0 = 10$, ×: $W_0 = 12.5 \sim 14$. – –: dashed line represents the value of $[TRI]_S$ when all feed TRI are solubilized.

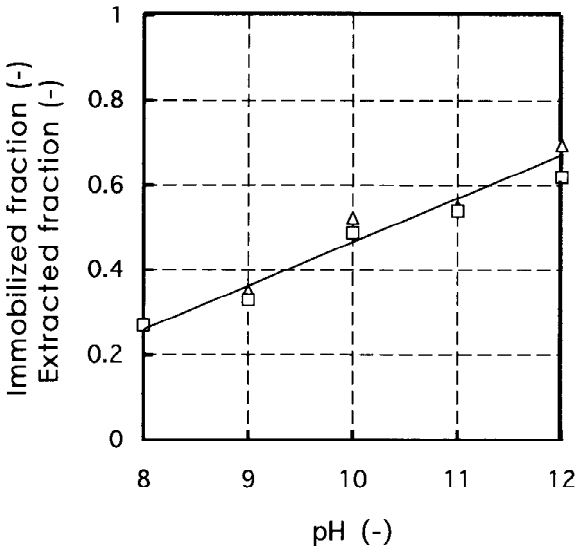

Figure 2. Effect of pH on immobilized fraction. $W_0 = 10$. △: immobilized fraction = $[\overline{TRI}]_{Im}/[\overline{TRI}]_S$ ($[\overline{TRI}]_S = 5.4 \times 10^{-5}M$, RC = 20). To check the denaturation of TRI at high pH, the extracted fraction was measured using the TRI immobilized at various pH. □: extracted fraction = $[\overline{P\text{-}TR}]/[P\text{-}TR]_F$ (feed concentration $[P\text{-}TR]_F = 1.8 \times 10^{-5}M$).

tion fraction is shown in Figure 3. When RC = 20, the immobilization fraction was around 0.6, but increased to over 0.9 when RC > 40.

Forward and Backward Extraction

We measured the extraction behavior for the P-*TR* from the aqueous phase to the micellar phase in order to check the extraction ability of the *TRI* immobilized at various pH. As shown in Figure 2, the extracted fraction of *TR* (= $[\overline{TR}]/[TR]_F$) increased with increasing the pH-value for the immobilization, indicating that the extraction ability of the immobilized *TRI* is retained up to higher pH range. Here, $[TR]_F$ represents the feed concentration of *TR* in the aqueous phase.

The extracted fraction of P-*TR* was also measured using the *TRI* immobilized at different RC-values (see Fig. 3). The obtained extracted fraction was above 0.9 in the range, RC > 40, indicating that the *TRI* decorated with cholesteryl groups retains its activity for *TR*-extraction up to at least 200 of RC.

Forward extraction for *TR* by the micelles with immobilized *TRI* proceeds by equation (1). We assume that some fraction α of the immobilized *TRI* is active irrespective of the number of combining choresteryl groups, and the inactive *TRI* is independent of the reaction.

$$TRI^* + TR = TRI \cdot TR,\ K = [\overline{TRI \cdot TR}]/([\overline{TRI}^*][TR]) \quad (1)$$

Here *K* is the equilibrium constant, and *TRI** represents the active *TRI*. The distribution coefficient *D* is defined by

$$D = [\overline{TRI \cdot TR}]/[TR] \quad (2)$$

The two conditions must be satisfied:

$$[\overline{TRI^*}] + [\overline{TRI \cdot TR}] = [\overline{TRI^*}]_{Im} = \alpha[\overline{TRI}]_{Im} \quad (3)$$

$$v_m[\overline{TRI \cdot TR}] = v_a([\overline{TR}]_F - [\overline{TR}]) \quad (4)$$

Here, $[\overline{TRI}]_{Im}$ stands for the total concentration of immobilized *TRI*, and v_m and v_a are the volumes of the micellar and aqueous phases, respectively. Because $v_m = v_a$ in this experiment, equations (1) to (4) provide:

$$[\overline{TRI}]_{Im}/D = 1/(\alpha K) + (1/\alpha)[TR]_F/(1 + D) \quad (5)$$

Figure 4 shows the relationship between $[\overline{TRI}]_{Im}/D$ and $[TR]_F/(1 + D)$. All data obtained under various experimental conditions locate around a straight line, which intercepts nearly zero at zero of $[TR]_F/(1 + D)$, indicating that *K* is

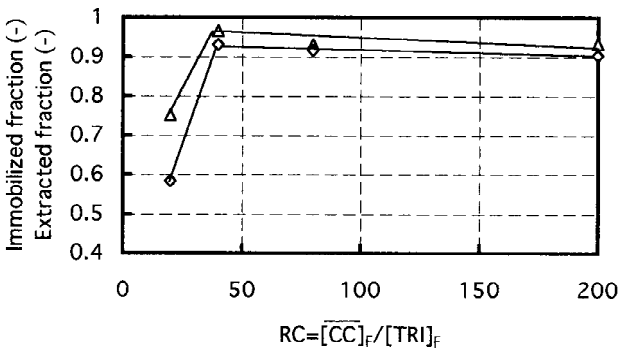

Figure 3. Effect of feed concentration ratio RC on immobilized fraction. $W_0 = 10$. pH of borate buffer = 12. ◇: immobilized fraction = $[\overline{TRI}]_{Im}/[\overline{TRI}]_S$ ($[TRI]_F = 1.9 \times 10^{-5}M$). To check the inactivation of TRI due to damage of the binding site, the extracted fraction was measured using the TRI immobilized at different RC-values. △: extracted fraction = $[\overline{P\text{-}TR}]/[P\text{-}TR]_F$ ($[P\text{-}TR]_F = 7.2 \times 10^{-6}M$).

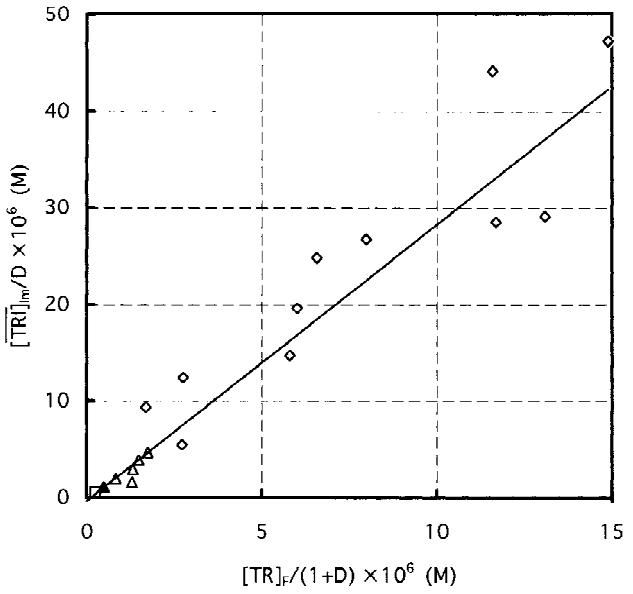

Figure 4. $[\overline{TRI}]_{Im/D}$ vs. $[TR]_F/(1 + D)$. Experimental conditions: $[\overline{TRI}]_{Im} = 0.8 \times 10^{-5}$ to $4.4 \times 10^{-5}M$, $[TR]_F = 0.7 \times 10^{-5}$ to $2.4 \times 10^{-5}M$, $[\overline{TRI}]_{Im}/[TR]_F = 1.2$ to 4.1 pH = 8. RC-values: ◇20, □40, △80, ▲200.

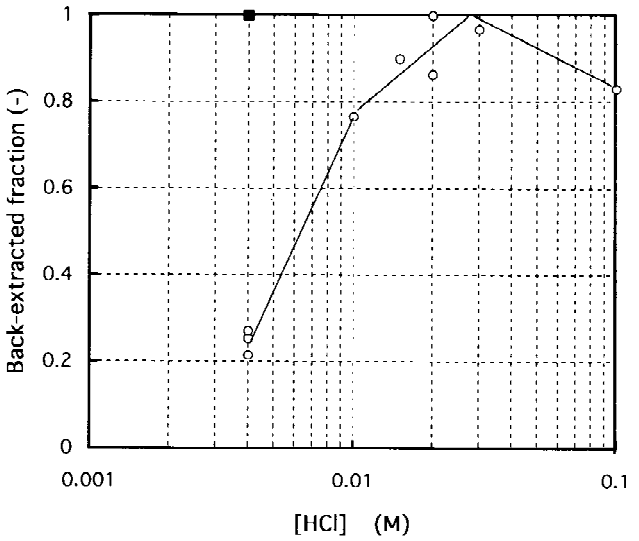

Figure 5. Effect of HCl concentration on back-extracted fraction. ■: B-TR, ○: P-TR.

very large. The slope of the straight line is about 2.8, i.e., $\alpha = 0.36$. The α-value is insensitive to the immobilization conditions.

The back-extraction behavior is shown in Figure 5. The back-extraction was almost completely attained for B-*TR* using 0.004*M* hydrochloric acid aqueous solution. The back-extracted fraction for P-*TR* was only 0.25 using the same aqueous solution. However, with increasing the concentration of hydrochloric acid, P-*TR* was easily back-extracted, and 100% back-extracted fraction was attained using the solution, [HCl] = 0.03*M*. To check the denaturation of P-*TR* by the strong acid, the enzymatic activity of the recovered P-*TR* was measured. The activity agreed with that of native P-*TR* when [HCl] < 0.015*M*, but decreased to around 60% of the activity of native P-*TR* when [HCl] > 0.02*M*. Because 90% back-extracted fraction was attained by 0.015*M* HCl, we used 0.015*M* HCl for back-extraction.

Extraction of Porcine Trypsin from Pancreatin

The extraction of P-*TR* from pancreatin is characterized by co-existence of large amounts of various contaminating proteins. The reverse micelles composed of nonionic surfactants have no specific interactions with water-soluble proteins, i.e., loss of the electrostatic and hydrophobic interactions. Thus, the driving force of the extraction is only the entropic effect, i.e., the dispersion of protein molecules to the water pools of the micelles. The amounts of proteins extracted by the entropic effect are usually very small. However, when the amounts of contaminating proteins are over several tens of a target protein, the contaminating proteins can not be neglected.

Because the target protein P-*TR* is bound tightly with the immobilized affinity ligand *TRI*, the contaminating proteins are expected to be easily removed by a simple washing

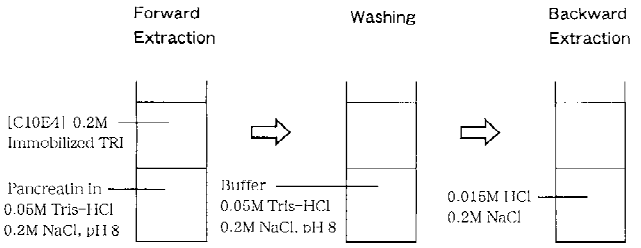

Figure 6. Procedure of selective separation of trypsin for pancreatin from porcine pancreas.

procedure. Thus, we tried the separation procedure shown in Figure 6. The immobilized *TRI* was obtained at the conditions; RC = 100 and pH = 12. The resultant liquid chromatograms for the washing aqueous solutions and for the final product after back-extraction are shown in Figure 7.

The original supernatant solution of pancreatin included a large amount of contaminating proteins with the retention time of less than 10 min and a small number with a retention time from 25 min to 45 min. The supernatant aqueous solution of pancreatin was contacted with the micellar phase. At this stage, a negligible amount of proteins with retention time of 25 to 45 min were extracted to the micellar phase. However, an appreciable amount of the components with

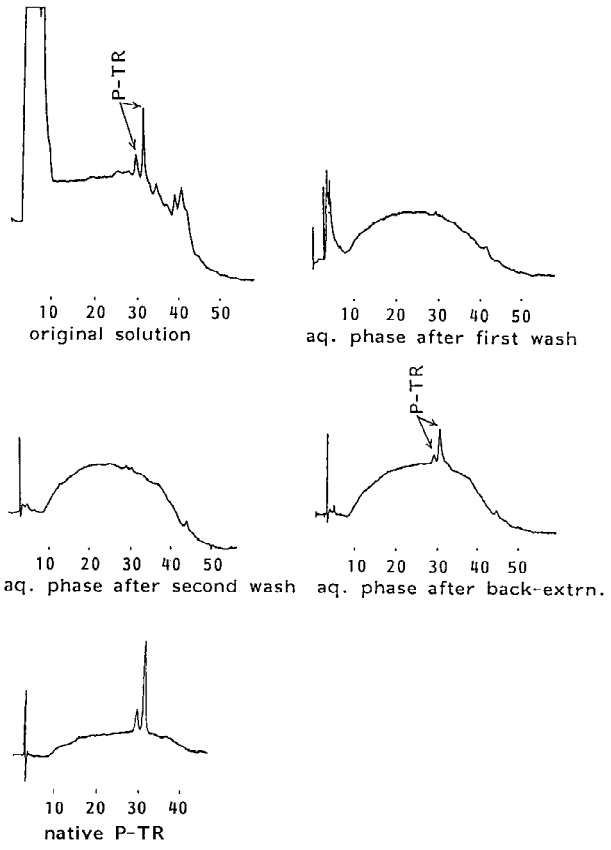

Figure 7. Liquid chromatograms in selective separation of TR from pancreatin. Liquid chromatogram of the native P-TR is also shown. The immobilized TRI was obtained at the conditions; RC = 100 and pH = 12. $[\overline{TRI}]_{Im} = 2.0 \times 10^{-5}M$.

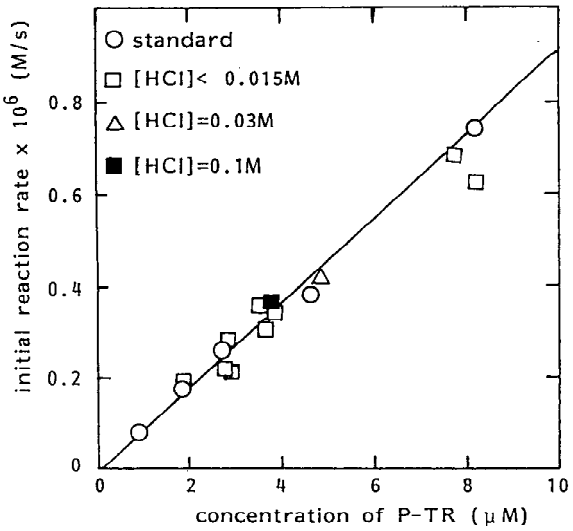

Figure 8. Activity of recovered TR from pancreatin. Method: hydrolysis rate of BAPNA.

the retention time of less than 10 min was extracted, because the aqueous phase after first washing did not contain the contaminating proteins with the retention time of from 25 min to 45 min, but contained those with the retention time of less than 10 min. After second washing no contaminating proteins were detected, indicating that the contaminating proteins were washed out completely by the first wash. No loss of the extracted P-*TR* was detected through the washing process. Finally, the P-*TR* in the reverse micellar phase was back-extracted by hydrochloric acid solutions. The aqueous solution after the backward extraction contained only P-*TR*, because the chromatogram after the backward extraction agreed with that of native P-*TR*. Thus, the selective separation of P-TR from many contaminating proteins was realized by the present method. The overall recovery of P-*TR* was around 35% to 50% at present.

The enzymatic activity of the recovered P-*TR* was measured from the hydrolysis rate of BAPNA as a function of P-*TR* concentration. As shown in Figure 8, the activity agreed with that obtained using standard P-*TR*, indicating that the P-*TR* in the product retains its enzymatic activity. Self-digestion for P-*TR* was not observed for the recovered P-*TR*. This is very advantageous for the separation of proteases. The use of the *TRI* as the affinity ligand in the forward extraction process and the acidic condition in the backward extraction process prevent the self-digestion of P-*TR*.

CONCLUSION

Selective separation of porcine trypsin from pancreatin was achieved without loss of activity of the recovered trypsin using trypsin inhibitor immobilized in reverse micelles, which were composed of a nonionic surfactant, tetraoxyethylenemonodecylether. The conditions for efficient operations throughout the whole separation processes were also shown. The active fraction of the immobilized trypsin inhibitor for extracting P-*TR* was 36% on average.

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