

Biotransformation of Amides to Acids Using a Co-Cross-Linked Enzyme Aggregate of *Rhodococcus erythropolis* Amidase

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Received: September 10, 2009 / Revised: October 6, 2009 / Accepted: October 7, 2009

Rhodococcus erythropolis amidase was expressed in *Escherichia coli* cells. The crude amidase in the cell-free extract was immobilized using the cross-linked enzyme aggregate (CLEA) method. The crude amidase was mixed with bovine serum albumin and then precipitated with ammonium sulfate. The resultant precipitant was subsequently cross-linked with glutaraldehyde. Scanning electron microscopy revealed that this co-CLEA had a ball-like shape with a diameter of approximately 1 μm . This co-CLEA evidenced hydrolytic activity toward a variety of amide substrates. The amidase co-CLEA evidenced an optimum temperature of 60°C and an optimum pH of 8.0, results that were similar to those of the soluble amidase. The reaction stability of the co-CLEA was increased. That is, it was stable up to 50°C and in a pH range of 5.0–12.0. Additionally, the co-CLEA could be recovered by centrifugation, and retained 96% activity after 3 repeated cycles. This amidase co-CLEA may prove useful as a substitute for soluble amidase as a biocatalyst in the pharmaceutical and chemical industries.

Keywords: Amidase, cross-linked enzyme aggregate, *Rhodococcus erythropolis*

Natural nitrile molecules are found in abundance on Earth, and many synthetic nitrile compounds are produced every day in the pharmaceutical and chemical industries [1]. These nitrile compounds are exploited by some microorganisms as carbon and energy sources. Two major nitrile-assimilation pathways have been identified [2]: one pathway utilizes nitrilase (E.C. 3.5.5.1) to convert nitriles directly to corresponding acids, and the other employs nitrile hydratase (NHase; E.C. 4.2.1.84) to hydrate nitriles into amides, and

amidase (E.C. 3.5.1.4) to hydrolyze amides into acids. These enzymes are critically important biocatalysts in the chemical and pharmaceutical industries [6, 20, 21].

There are many methods for enzyme immobilization on rigid supports: namely, covalent immobilization [8], ionic immobilization [11, 18], and hydrophobic adsorption [13]. However, methods for the immobilization of enzymes without the use of supports are gaining in importance because they offer the advantages of high volumetric productivity and lower production costs. In recent years, it has been demonstrated that the chemical cross-linking of enzyme precipitates generates quite robust biocatalysts [3, 12, 16]. The preparation of these cross-linked enzyme aggregates (CLEA) involves protein precipitation followed by cross-linking with glutaraldehyde. This method is attractive in its simplicity and robustness, as well as for the possibility of using a semipurified enzyme and the opportunity to co-immobilize two different enzymes. However, when the proteins are very poor in external lysine residues, the preparation of CLEA may become a far more complicated process [10, 16, 19]. This problem may be partially solved by coprecipitating the enzyme and some polymers containing a large number of primary amino groups, such as polyethyleneimine [9].

In this study, *R. erythropolis* amidase was utilized for the preparation of CLEA. This enzyme evidences low lysine residue content, which prevents the intermolecular cross-linking between the enzymes. This problem was solved by the addition of bovine serum albumin (BSA) as a co-CLEA protein. The biochemical properties of this amidase/BSA co-CLEA are characterized in this study.

MATERIALS AND METHODS

Chemicals

Acetamide, acrylamide, benzamide, butyramide, isobutyramide, and propionamide were purchased from Sigma-Aldrich Inc. (St. Louis,

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MO, U.S.A.). Bovine serum albumin and glutaraldehyde were obtained from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). 4-Chloro-3-hydroxybutyronitrile (CHBNitrile) was supplied by Equispharm, Inc. (Korea). 4-Chloro-3-hydroxybutyramide (CHBamide) was prepared using CHBNitrile, as previously described [14].

Production of *R. erythropolis* Amidase in *E. coli*

Recombinant *E. coli* BL21 (DE3)/pET22-ami with the *R. erythropolis* amidase gene [14] was cultured at 18°C in LB medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl, per liter) containing 100 µg/ml of ampicillin. When the OD₆₀₀ reached 0.5, IPTG was added to a final concentration of 1 mM and cultured further for an additional 20 h. The cultured cells were harvested by centrifugation (6,000 ×g, 10 min) and resuspended in a potassium phosphate buffer (10 mM, pH 7.5). The cells were disrupted by sonication and the soluble fraction was obtained by centrifugation (10,000 ×g, 15 min). This cell-free extract was utilized as a crude amidase for the preparation of CLEA.

Amidase Activity Assay

The amidase activity assay was performed by measuring the reaction product, NH₃, using the method developed by Fawcett and Scott [7]. A small amount of amidase CLEA was added to 1 ml of 10 mM potassium phosphate buffer (pH 7.5) containing 5 mM isobutyramide, and the reaction mixtures were incubated for 30 min at 40°C. One hundred µl of reaction mixture was sampled and mixed with 0.5 ml of solution A (10 g of phenol and 0.4 g of sodium nitroprusside per liter) and 0.5 ml of solution B (5 g of NaOH and 7 ml of sodium hypochlorite solution per liter). After 30 min of incubation at room temperature, the OD₆₂₅ was measured and compared with a NH₄OH (0–0.5 mM) standard curve. One unit of enzyme activity was defined as the quantity of enzyme necessary to generate 1 µmole of NH₃ per minute.

Preparation of Amidase Co-CLEA

Amidase CLEA was prepared using the crude amidase alone, whereas amidase co-CLEA was prepared using the same crude amidase together with BSA protein. Ammonium sulfate was added to a 70% saturation into the crude amidase solution (100 mg total protein/10 ml volume). The physically aggregated protein was then subjected to chemical cross-linking using 2 ml of glutaraldehyde (25% aqueous solution). This suspension was stirred for 20 h at 4°C. The mixture was centrifuged for 10 min at 6,000 ×g. The supernatant was decanted and the pellets were washed three more times with potassium phosphate buffer (10 mM, pH 7.5) to remove the unreacted glutaraldehyde. The washed CLEAs were resuspended in the same buffer and stored at 4°C.

To compare the efficiency of the above method, 1,2-dimethoxy ethane (DME) was utilized as a precipitant rather than ammonium sulfate. Two volumes of DME were added to the crude amidase solution, after which the resultant precipitant was treated by the same procedure as described above.

To prepare the amidase co-CLEA, 100 mg of the crude amidase and different amounts (50, 100, and 150 mg) of BSA were mixed first in 10 ml of solution. Then, the CLEA preparation process was performed, as described above.

The shape and surface morphology of the co-CLEA was assessed using a scanning electron microscope (model JSM 5410 LV; JEOL, Tokyo, Japan) after the samples were dried at ambient temperature,

and then coated with gold. The particle size of the co-CLEA was determined using a Zetasizer Nano ZS (model ZEN3600; Malvern, U.K.).

Biotransformation of Various Amides Using Amidase Co-CLEA

Short-chain amides (acetamide, acrylamide, and propionamide), mid-chain amides (butyramide, 4-chloro-3-hydroxybutyramide, and isobutyramide), and an aromatic amide (benzamide) were utilized as substrates for amidase co-CLEA. Enzyme reactions using 5 mM of each substrate and NH₃ quantification were conducted in accordance with the standard assay method described previously.

Effects of Temperature and pH on Co-CLEA

To determine the optimum temperature, amidase co-CLEA was added to 1 ml of 10 mM potassium phosphate buffer (pH 7.5) containing 5 mM isobutyramide, and the reaction mixtures were incubated for 30 min at various temperatures (10–70°C), after which the amount of NH₃ produced was measured. To determine the temperature stability, amidase co-CLEA was incubated for 30 min at various temperatures (10–70°C) and its residual activity was measured by the standard assay method.

To determine the optimum pH, we conducted an amidase assay with various pH buffers including sodium acetate buffer (pH 4–6), KH₂PO₄–K₂HPO₄ buffer (pH 6–9), and K₂HPO₄–K₃PO₄ buffer (pH 9–12). To determine the pH stability, amidase co-CLEA was incubated for 30 min with the above pH buffers, and its residual activity was measured by the standard assay method.

Stability of Amidase Co-CLEA in Organic Solvents

To determine the effects of organic solvents on the CLEA, an amidase assay was conducted in the presence of various concentrations (10–70%) of each water-miscible organic solvent, including methanol, ethanol, acetone, DMSO, and acetonitrile.

Reusability of Amidase Co-CLEA

To assess the reusability of amidase co-CLEA, the CLEA was washed with the assay buffer after each reaction and then suspended in a fresh reaction mixture to evaluate the residual activity. The same reusability test was conducted for the intact *R. erythropolis* and *E. coli* cells. The residual activity was calculated by establishing each initial amidase activity as 100%.

Hydrolysis of (*R*)- and (*S*)-CHBamide Using Amidase Co-CLEA

The reaction mixture (2 ml) containing 0.53 ml of amidase co-CLEA (0.6 U), 0.1 ml of (*R*)- or (*S*)-CHBamide (28 mg), and 1.37 ml of potassium phosphate buffer (10 mM, pH 7.5) was prepared and incubated at 40°C. At various intervals, 30-µl portions of the reaction mixture were sampled and their NH₃ contents were measured.

RESULTS AND DISCUSSION

Preparation of Co-Cross-Linked Amidase Aggregate

The *R. erythropolis* amidase gene (pET22-ami) [14] was expressed in *E. coli* BL21 (DE3) cells, and cell-free extracts of the recombinant *E. coli* cells were utilized as a crude amidase. Amidase CLEA was prepared using the crude amidase according to the typical CLEA preparation method,

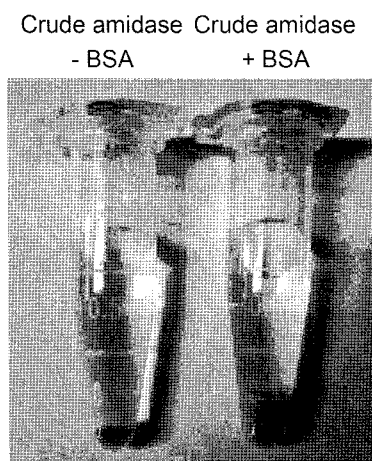


Fig. 1. Preparation of amidase co-CLEA. The two microtubes show the CLEAs prepared in the absence or presence of BSA, respectively.

as previously described [5, 17]. However, the yield was quite low (less than 7% of the original amidase activity) (Fig. 1 and Table 1). The crude amidase was mixed with various quantities of BSA and precipitated with ammonium sulfate, after which the cross-linking reaction was conducted in accordance with the CLEA preparation procedure described above. In this case, the yield was increased up to 57% of the original amidase activity (Fig. 1 and Table 1), thus showing that this co-CLEA preparation might be employed as an amidase-immobilization method. This result can be explained by the fact that the *R. erythropolis* amidase protein evidenced relatively low lysine content (3.6%) and thus had too few surface amino groups to form cross-linkages with one another. It seemed that the degree of the cross-linking of amidase protein was greatly increased in number with the help of the lysine-rich BSA protein (9.9% lysine content) (Fig. 1).

The BSA added to the crude amidase solution exerted different effects on CLEA formation, according to its abundance. Crude amidase (100 mg total protein) was mixed with 50, 100, and 150 mg of BSA, and each mixture

was used for CLEA formation. The resultant CLEA yields were measured as 57%, 56%, and 34%, respectively, showing that BSA had different effects on the cross-linking process of CLEA formation (Table 1).

When DME was utilized as another protein precipitant instead of ammonium sulfate, the CLEA yield was not more than 33%. Regardless of ammonium sulfate or DME, the amidase activities after precipitation proved to be similar. Therefore, the relatively low yield might be the result of low cross-linking efficiency.

Observation by scanning electron microscopy demonstrated that those amidase co-CLEAs exhibited a ball-like morphology (Fig. 2A), similar to other previously reported CLEAs [15]. The average particle sizes of each co-CLEA were measured as 1.23, 0.91, and 1.69 μm , respectively (Fig. 2B), by the Zeta potential measurement, which were also similar to those of other CLEA particles [15].

Transformation of Amides to Acids Using Amidase Co-CLEA

The hydrolytic activity of amidase co-CLEA toward a variety of amide substrates was measured (Table 2). As in the case of the soluble amidase, amidase co-CLEA was shown to be capable of hydrolyzing a variety of amide substrates. Isobutyramide, butyramide, and propionamide were found to be hydrolyzed more rapidly than acetamide, acrylamide, and benzamide. These results indicated that amidase co-CLEA might prove useful in the biotransformation reaction, as a substitute for the soluble amidase.

Biochemical Properties of Amidase Co-CLEA

Because isobutyramide was hydrolyzed most rapidly, it was utilized as a standard substrate in the following experiments. The optimum temperature of the amidase co-CLEA was measured as 60°C, which is 10°C higher than that of soluble amidase (Fig. 3A). The amidase co-CLEA evidenced higher thermostability in comparison with the soluble enzyme; amidase co-CLEA evidenced 60% activity at 60°C, whereas the soluble enzyme exhibited no activity at 60°C (Fig. 3B). The optimum pH of the amidase co-

Table 1. Amidase activity of the amidase/BSA co-CLEA.

Precipitant	BSA (mg/100 mg crude amidase)	Amidase activity after precipitation (U)	Amidase activity after CLEA formation (U)	Recovery (%)
Ammonium sulfate	0	16.4	1.7	7
	50	17.7	14.5	57
	100	22.8	14.2	56
	150	23.4	8.6	34
1,2-Dimethoxy ethane	0	20.1	1.3	5
	50	29.1	3.7	15
	100	21.4	5.1	20
	150	25.3	8.3	33

The starting amidase activity was 25.3 units/100 mg crude amidase preparation.

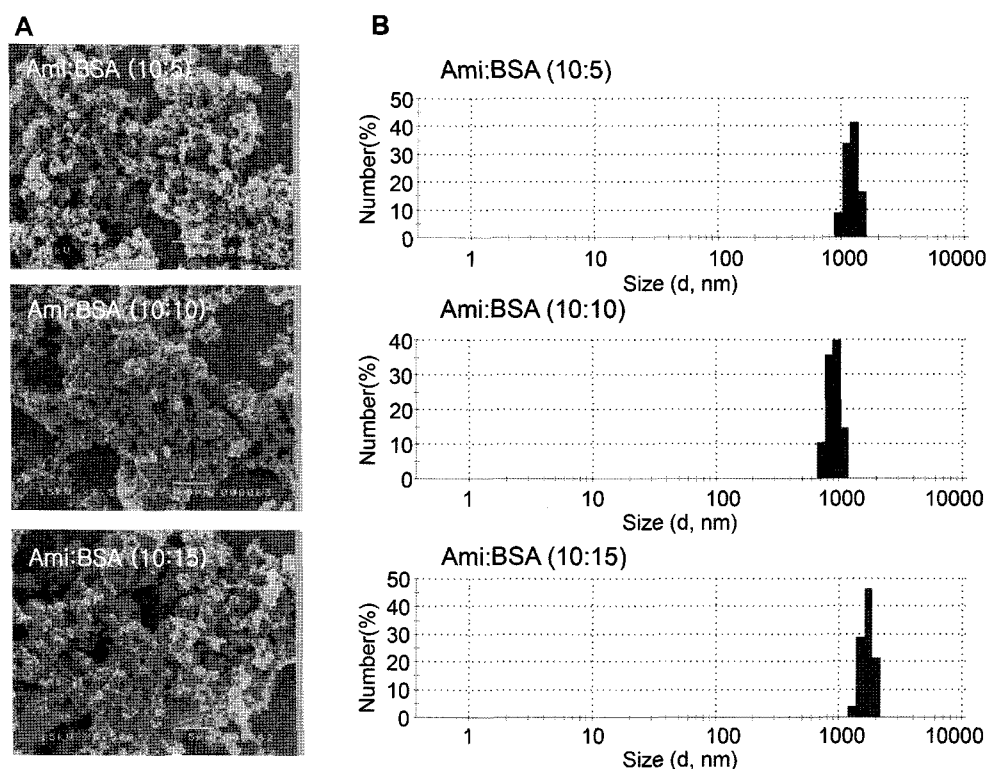


Fig. 2. Morphology of amidase co-CLEA.

A. The CLEA prepared from the crude amidase and BSA was pictured by SEM. B. The size of the CLEA was measured with a Zetasizer Nano ZS.

CLEA was determined to be pH 8.0, which was similar to that of the soluble enzyme (Fig. 3C). On the other hand, the pH stability of the amidase co-CLEA differed from that of the soluble one; it was relatively stable at a broader pH range of 5.0–12.0, whereas the soluble enzyme proved stable at a pH range of 7.0–11 (Fig. 3D).

The activity of amidase co-CLEA in a variety of solvents was assessed and the results are provided in Fig. 3F. As the concentration of acetonitrile, acetone, and DMSO increased

to higher than 10%, the amidase activity decreased dramatically. The amidase co-CLEA maintained more than 50% of its original activity in the presence of 20% methanol or 40% ethanol. As such, the activity of the amidase co-CLEA was similar or rather increased depending on the organic solvents, in comparison with the soluble enzyme.

The reusability of the amidase co-CLEA was assessed and compared with those of *R. erythropolis* cells and recombinant *E. coli* cells by measuring their residual activity

Table 2. Hydrolytic activity of the co-CLEA toward various amide substrates.

Substrate	R group	Amidase co-CLEA		Soluble amidase	
		Activity (U/ml)	Relative activity (%)	Activity (U/ml)	Relative activity (%)
Acetamide	CH ₃ -	0.50	12	1.39	5
Acrylamide	CH ₂ =CH-	0.73	18	2.75	10
Benzamide	C ₆ H ₅ -	1.25	30	5.41	20
Butyramide	CH ₃ CH ₂ CH ₂ -	2.19	53	10.94	41
CHBamide ^a	ClCH ₂ CH(OH)CH ₂ -	1.65	40	10.12	38
Isobutyramide	CH ₃ (CH ₃)CH-	4.14	100	25.56	100
Propionamide	CH ₃ CH ₂ -	2.55	62	10.71	40

^aCHBamide: 4-chloro-3-hydroxy butyramide.

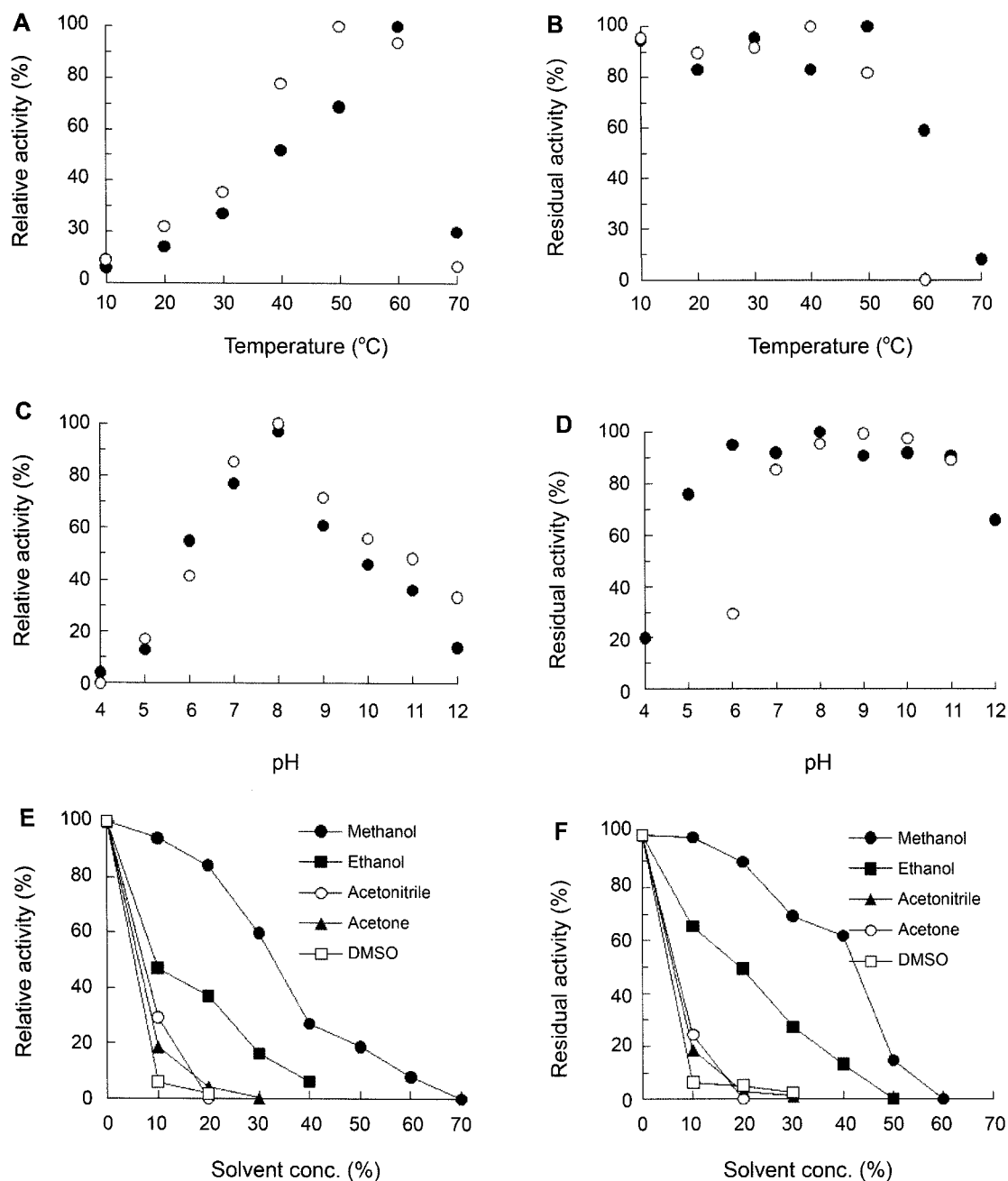


Fig. 3. Properties of the amidase co-CLEA and soluble amidase.

The effects of temperature on the amidase activity (A) and stability (B) were measured. The effects of pH on the amidase activity (C) and stability (D) were measured. Open circle (○) and closed circle (●) represent the soluble amidase and amidase co-CLEA, respectively. The effects of organic solvents on the soluble amidase (E) and amidase co-CLEA (F) were measured.

after several recovery processes (Fig. 4). Even after 3 repeated cycles, amidase co-CLEA maintained similar activity with the original samples. However, the amidase activities of the *R. erythropolis* cells and recombinant *E. coli* cells decreased gradually as the recovery cycles were repeated. After 3 repeated cycles, the retained amidase activities of the *R. erythropolis* [14] and recombinant *E. coli* cells [14] were 39% and 15% of each original activity,

respectively. Of course, the soluble amidase enzyme could not be recovered by centrifugation.

Hydrolysis of CHBamide

The amidase co-CLEA was found to be capable of converting racemic CHBamide into CHB acid (Table 2), which has been identified as an important intermediate in the synthesis of chiral drugs [4, 14]. When (*R*)-CHBamide

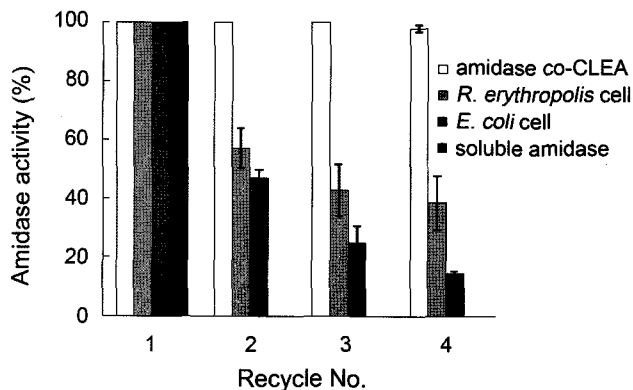


Fig. 4. Reusability of amidase co-CLEA.

The amidase activities of the amidase co-CLEA, *R. erythropolis*, and *E. coli* BL21 (DE3)/pET22-ami were measured several times after repeated washings.

or (*S*)-CHBamide was used as a substrate, the amidase co-CLEA hydrolyzed the (*R*)-form more rapidly than the (*S*)-one (Fig. 5B). The enantiomeric excess (e.e.) value was calculated as 60% when the conversion yield reached

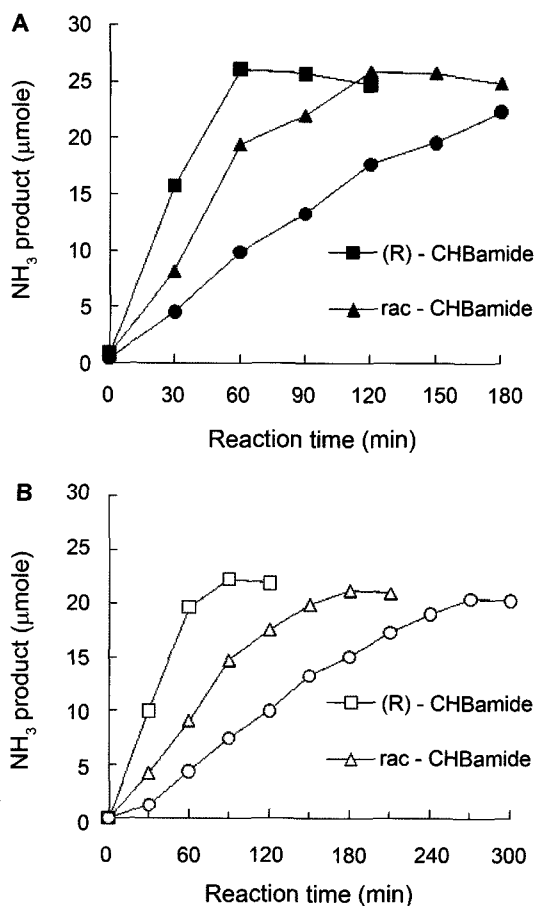


Fig. 5. Hydrolysis of CHBamide.

The hydrolysis rates of racemic CHBamide, (*R*)-CHBamide, and (*S*)-CHBamide with the soluble enzyme (A) and amidase co-CLEA (B) were measured.

65%. This preference of the amidase co-CLEA for (*R*)-CHBamide was similar to that observed with the soluble amidase (Fig. 5A).

Collectively, the results of this study showed that *R. erythropolis* amidase could be immobilized together with a lysine-rich second protein such as BSA, and that the substrate specificity and enantioselectivity of the amidase co-CLEA were only slightly changed in the cross-linking process. Instead, the temperature, pH, and organic solvent stability values increased substantially. Accordingly, this amidase co-CLEA may be widely utilized as a substitute for the soluble enzyme in the pharmaceutical and chemical industries.

Acknowledgments

This work was supported by the 21C Frontier Microbial Genomics and Applications Center Program, Ministry of Education, Science and Technology, Republic of Korea and by the Gyeonggi Regional Research Center (GRRC) at the Catholic University of Korea.

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