Purification of Arachidonic Acid from *Mortierella* Single-Cell Oil by Selective Esterification with *Burkholderia cepacia* Lipase

Asao Yamauchi[a,*], Toshihiro Nagao[a], Yomi Watanabe[a], Motoo Sumida[b], Takashi Kobayashi[a], and Yuji Shimada[a]

[a]Osaka Municipal Technical Research Institute, Osaka 536-8553, Japan, and [b]Institute for Health Care Science, Suntory Ltd., Osaka 618-0001, Japan

ABSTRACT: Purification of arachidonic acid (AA) from *Mortierella alpina* single-cell oil was attempted. The process comprised three steps: (i) preparation of FFA by nonselective hydrolysis of the oil with *Alcaligenes* sp. lipase; (ii) elimination of long-chain saturated FA from the resulting FFA by urea adduct fractionation; and (iii) enrichment of AA through lipase-catalyzed selective esterification with lauryl alcohol (LauOH). In the third step, screening of industrially available lipases indicated that *Burkholderia cepacia* lipase (Lipase-PS, Amano Enzyme Inc., Aichi, Japan) acted on AA more weakly than on other FA and was the most effective for enrichment of AA in the FFA fraction. When the FFA obtained by urea adduct fractionation were esterified with 2 molar equivalents of LauOH at 30°C for 16 h in a mixture with 20% water and 20 units (U)/g-mixture of Lipase-PS, the esterification reached 39% and the content of AA in the FFA fraction was raised from 61 to 86 wt%. To further increase the content of AA, unesterified FFA were allowed to react again under the same conditions as those in the first selective esterification except for the use of 50 U/g Lipase-PS. A series of procedures raised the content of AA to 97 wt% with a 49% recovery based on the initial content in the single-cell oil. These results indicated that the three-step process for selective esterification with Lipase-PS was effective for purifying AA from the single-cell oil.

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KEY WORDS: *Alcaligenes*, arachidonic acid, *Burkholderia cepacia*, esterification, hydrolysis, lipase, *Mortierella alpina*, purification, single-cell oil, urea adduct fractionation.

Arachidonic acid (AA; 20:4n-6) is a precursor of local hormones (prostagrandins, leukotrienes, and thromboxanes) in the human body, and plays a regulatory role in a number of biological functions (1,2). Dietary AA is also known to improve the growth of infants (3,4) and the brain functions of the elderly (5). In addition, derivatives of AA, such as anandamide (arachidonoylethanolamide) and 2-arachidonylglycerol, are endogenous ligands for cannabinoid receptors and are expected to have potential medical benefits (6). Owing to these physiological activities, AA is currently receiving increased attention in the pharmaceutical and food industries.

*To whom correspondence should be addressed at Osaka Municipal Technical Research Institute, 1-6-50 Morinomiya Joto-ku, Osaka 536-8553, Japan. E-mail: yamauchi@omtri.city.osaka.jp

AA is a rare FA; its natural sources are mainly animal viscera and marine algae, with contents of AA <0.5% on a dry weight basis (7). Efforts to produce AA efficiently have succeeded in the industrial production of a single-cell oil containing >40 wt% AA by fermentation of *Mortiella alpina* (8,9). Dietary AA of higher purity is, however, strongly desired for its application as an additive in foods and as a nutritional supplement (10). Argentated silica gel column chromatography was recently reported to be effective for the purification of AA, and the purity was raised to 97 wt% when a single-cell oil containing 35 wt% AA was used as a starting material (11). However, this method has not been adopted as an industrial process because of the residual silver and high cost; thus, development of a suitable method is expected.

To date, we have reported the enrichment of AA from a single-cell oil by a three-step process (12,13). The first step was the preparation of FFA by nonselective hydrolysis of the oil containing AA with *Burkholderia cepacia* lipase; the second step was elimination of long-chain saturated FA by urea adduct fractionation; and the third step was enrichment of AA in the FFA fraction through selective esterification of FFA with lauryl alcohol (LauOH) using *Candida rugosa* lipase, which acted weakly on AA. However, the purity of the resulting preparation was not sufficiently high (81 wt%). Because *C. rugosa* lipase acted on γ-linolenic acid (GLA, 18:3n-6) and dihomo-γ-linolenic acid (DGLA, 20:3n-6) as weakly as on AA, not only AA but also GLA/DGLA were enriched in the FFA fraction (12–14).

This study shows that *B. cepacia* lipase acted on GLA and DGLA more strongly than on AA, and that AA can be highly purified by the lipase-catalyzed esterification of FFA originating from a single-cell oil containing AA with LauOH.

MATERIALS AND METHODS

Materials. TGA40 oil containing 44 wt% AA was a commercial product of Suntory Ltd. (Osaka, Japan). LauOH was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). The lipases were gifts from the following companies: *B. cepacia* lipase (Lipase-PS, originally classified as *Pseudomonas* sp. lipase) and *Rhizopus oryzae* lipase (Lipase-T) were from Amano Enzyme Inc. (Aichi, Japan); *Alcaligenes* sp. lipase (Lipase-QLM), *C. rugosa* lipase (Lipase-OF), *B. cepacia* lipase

(Lipase-SL, originally classified as *Pseudomonas cepacia* lipase), and *Pseudomonas stutzeri* lipase (Lipase-TL) were from Meito Sangyo Co. Ltd. (Aichi, Japan); *Pseudomonas aeruginosa* lipase (Lipase-LPL) was from Toyobo Co. Ltd. (Osaka, Japan). Because the origin of Lipase-PS and -SL was from *B. cepacia*, the two lipases are hereafter referred to as Lipase-PS and -SL, respectively. One unit (U) of lipase activity was defined as the amount of lipase that liberated 1 μmol of FA per minute in the hydrolysis of olive oil as described previously (15).

Preparation of FFA from TGA40 oil. In a typical procedure, TGA40 oil was hydrolyzed at 40°C for 48 h in a mixture containing 67% water and 1200 U/g-mixture of *Alcaligenes* sp. lipase with stirring at 500 rpm under a nitrogen atmosphere. The degree of hydrolysis was calculated from the acid value of the reaction mixture and the saponification value (184 mg KOH/g) of the original TGA40 oil. After the hydrolysis reaction, FFA were extracted from the reaction mixture with *n*-hexane as described previously (16). In brief, 0.5 N KOH/10% ethanol was added to the reaction mixture, and undigested acylglycerols were removed with *n*-hexane. FFA in the water layer were then extracted with *n*-hexane after returning to an acidic pH (<pH 2) with HCl.

Urea adduct fractionation. Urea adduct fractionation was performed in a manner similar to that described previously (17). FFA (50 g) originating from TGA40 oil was dissolved at 60°C in a solution of 250 mL methanol, 6.8 mL water, and 50 g urea. The temperature was decreased gradually to 4°C with stirring. After the resulting precipitate was removed by filtration, 300 mL of 0.1 N HCl was added to the filtrate, and FFA were then extracted with *n*-hexane. Finally, the organic solvent was removed by evaporation.

Selective esterification. Unless otherwise specified, esterification of FFA with LauOH was conducted under the following conditions: A mixture of 4 g FFA/LauOH (1:2, mol/mol), 1 g water, and various amounts of lipase was incubated at 30°C for 16 h with stirring at 500 rpm under a nitrogen atmosphere. The acid value was measured before and after the reaction by titrating with 1.0 N KOH, and the degree of esterification was calculated on the basis of the amount of FFA consumed during the reaction. After the reaction, unesterified FFA were extracted from the reaction mixture with *n*-hexane in the aforementioned manner (16).

Analyses. FFA prepared by extraction with *n*-hexane were methylated in 3 mL of 5% HCl/methanol at 75°C for 10 min. FA in TGA40 oil were methylated at 75°C in 3 mL methanol containing 1% sodium methylate for 15 min. The resulting FAME were analyzed with an Agilent 6890N gas chromatograph (Palo Alto, CA) connected to a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) as described previously (18). The initial column temperature was 150°C, which was increased by 4°C/min to 170°C, 5°C/min to 195°C, and 10°C/min to 215°C, followed by a hold at that temperature for 11 min. The injector and detector temperatures were set at 245 and 250°C, respectively. The split ratio was 100:1, and helium was used as the carrier gas with a constant flow rate of 0.8 mL/min. The contents of LauOH and FA lauryl esters were analyzed with a Shimadzu GC-18A gas chromatograph (Kyoto, Japan) connected to a DB-1ht capillary column (0.25 mm × 5 m; J&W Scientific). The column temperature was raised from 100 to 300°C at 15°C/min, and the injector and detector temperatures were set at 370 and 390°C, respectively. The split ratio was 50:1, and helium was used as the carrier gas at 1.0 mL/min.

RESULTS AND DISCUSSION

Hydrolysis of TGA40 oil. Lipase-PS was used previously for the nonselective hydrolysis of TGA40 oil, but the degree of hydrolysis was <80% (12,13). Hence, several lipases from *Burkholderia*, *Pseudomonas*, and *Alcaligenes* were screened to find a lipase suitable for hydrolysis of the single-cell oil. These lipases were known to act on PUFA, although their activities were weak compared with those on C_{18} FA (13,19). The screening test showed that *Alcaligenes* sp. lipase (Lipase-QLM) attained >98% hydrolysis after 48 h but the degree of hydrolysis by the other lipases (Lipases-PS, -SL, -TL, and -LPL) was <84%. The *Alcaligenes* sp. lipase was therefore selected, and the reaction conditions were fixed as follows: ratio of TGA40/water, 1:2 (w/w); amount of lipase, 1200 U/g-mixture; temperature, 40°C.

TGA40 oil was hydrolyzed by *Alcaligenes* sp. lipase under the determined conditions (Fig. 1). To show the superiority of this lipase, Lipase-PS, which was used in our previous report (12,13), was also used as a catalyst. The initial velocity of the hydrolysis by *Alcaligenes* lipase was almost the same as that by Lipase-PS. But the hydrolysis by *Alcaligenes* lipase proceeded efficiently even after 6 h, and the degree of hydrolysis reached 98% after 48 h. Meanwhile, the degree of hydrolysis in the reaction with Lipase-PS did not exceed 83% even after 72 h.

FFA were prepared from the 48-h hydrolysate by extraction with *n*-hexane: The resulting FFA mixture was named FFA-

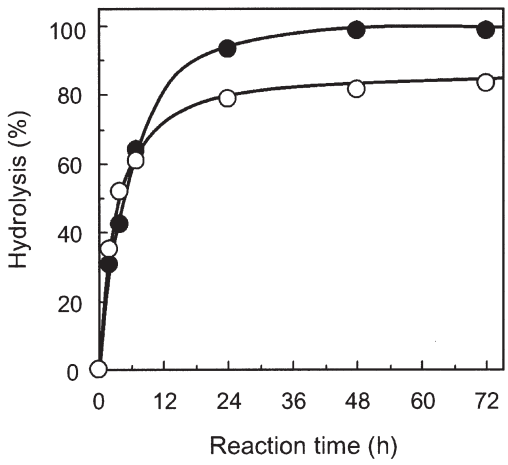

FIG. 1. Time course of hydrolysis of TGA40 oil (Suntory Ltd., Osaka, Japan) with lipases from *Alcaligenes* sp. and *Burkholderia cepacia* (Lipase-PS). A mixture of 3 g TGA40 oil, 6 g water, and 1200 U/g-mixture of lipase was stirred at 40°C. (●) Hydrolysis with *Alcaligenes* sp. lipase; (○), Lipase-PS.

TABLE 1
Extent of Esterification of FFA-Urea with Lauryl Alcohol (LauOH) Using Various Lipases[a]

Lipase	Degree of esterification (%)			
	Total FA[b]	18:3[c]	20:3[c]	20:4[c] (AA)
Burkholderia cepacia[d]	35.3	89.7	68.3	7.1
Alcaligenes sp.	33.9	83.9	53.2	11.9
Burkholderia cepacia[e]	30.8	72.8	42.1	14.0
Pseudomonas aeruginosa	22.8	57.2	20.3	9.6
Pseudomonas stutzeri	20.2	30.3	14.8	17.9
Rhizopus oryzae	29.3	22.1	19.7	23.9
Candida rugosa	30.0	20.3	20.2	18.4

[a]FFA-Urea contained 3.3 mol% γ-linolenic acid, 4.4 mol% dihomo-γ-linolenic acid, and 59.1 mol% arachidonic acid (AA). A mixture of 4 g FFA-Urea/LauOH (1:2, mol/mol), 1 g water, and 200 units (U)/g-mixture of lipase was stirred at 30°C for 2 h.
[b]The esterification was calculated from the acid value of the reaction mixture before and after the reaction.
[c]Molar ratio of the amount of each FA in the FA lauryl ester fraction to that of the FA in FFA-Urea.
[d]Lipase-PS (Amano Enzyme Inc., Aichi, Japan).
[e]Lipase-SL (Meito Sangyo Co. Ltd., Aichi, Japan).

Hyd. The FA composition in FFA-Hyd was almost the same as that of the original TGA40 oil, and the content of AA was 45 wt%. This result indicated that the *Alcaligenes* sp. lipase acted on AA as strongly as on the other constituent FA under the determined reaction conditions.

Urea adduct fractionation. FFA-Hyd was in a solid state at room temperature because of the high content of saturated FA, such as behenic acid (22:0; 3 wt%) and lignoceric acid (24:0; 9 wt%). In general, lipase reactions proceeded efficiently on liquid-state substrates but not on solid-state ones. FFA-Hyd was therefore subjected to urea adduct fractionation prior to selective esterification for the removal of long-chain saturated FA. The fractionation removed almost all behenic and lignoceric acids, and a part of the palmitic acid (16:0) and stearic acid (18:0). The resulting FFA mixture was in a liquid state, and the content of AA increased from 45 to 61 wt%: The FFA mixture was named FFA-Urea.

Suitable lipase for selective esterification. Our previous studies showed that LauOH was the most effective for the lipase-catalyzed selective esterification because LauOH shifted the equilibrium to esterification even in the presence of 20 wt% water (12,20). The following esterification of FFA-Urea was therefore conducted using LauOH as a substrate in the presence of 20 wt% water.

To study the activities of several lipases toward AA, GLA, and DGLA, FFA-Urea was esterified at 30°C for 2 h with 2 molar equivalents of LauOH using 200 U/g-mixture of enzyme (Table 1). The degree of esterification of the total FA reached 20–35% under the fixed reaction conditions. The *C. rugosa* and *R. oryzae* lipases esterified AA, GLA, and DGLA to a similar degree, although the activities were weak. However, two *B. cepacia* lipases and the *Alcaligenes* sp. lipase esterified AA more weakly than GLA and DGLA. In particular, Lipase-PS discriminated the most efficiently between AA and GLA/DGLA. These results indicated that Lipase-PS acted on FA with C_{18} to C_{20} carbon lengths, but weakly on FA in which an unsaturated bond existed between the carboxyl group and the fifth carbon from the carboxyl group, or in which the number of unsaturated bonds was four or more. In addition, the lipase acted on palmitic, stearic, linoleic, and oleic acids as strongly as on GLA (data not shown). Hence, Lipase-PS was chosen as the catalyst in the selective esterification of FFA-Urea for enrichment of AA.

Effects of amounts of LauOH and lipase on selective esterification. FFA-Urea was esterified at 30°C for 16 h with 1–7 molar equivalent(s) of LauOH in a mixture containing 20 wt% water and 40 U/g-mixture of Lipase-PS (Fig. 2). The degree of esterification was the highest at 2 molar equivalents of LauOH to FFA-Urea. Because the content of AA in the FFA fraction also correlated with the degree of esterification (the content of AA at 2 molar equivalents of LauOH, 90 wt%), the ratio of LauOH to FFA-Urea was fixed at 2:1 (mol/mol).

To study the effect of the amount of lipase on the esterification, FFA-Urea was esterified at 30°C for 16 h with 2 molar equivalents of LauOH in a mixture containing 20 wt% water and

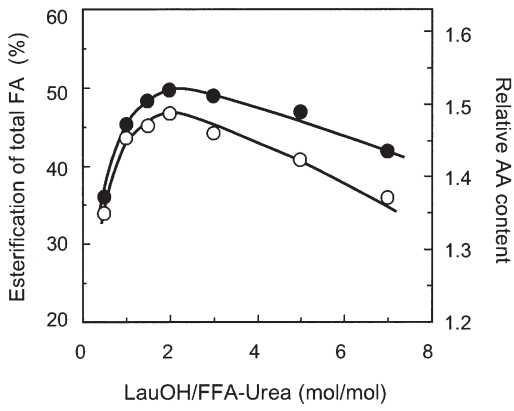

FIG. 2. Effect of the amount of lauryl alcohol (LauOH) on selective esterification of FFA-Urea with Lipase-PS. A 5-g mixture of FFA-Urea, various amounts of LauOH, 20 wt% water, and 40 U/g-mixture of lipase was stirred at 30°C for 16 h. (●) Degree of esterification of total FA; (○) content of arachidonic acid (AA) in the FFA fraction. The content was expressed relative to that in FFA-Urea (61.1 wt%). For other abbreviation see Figure 1.

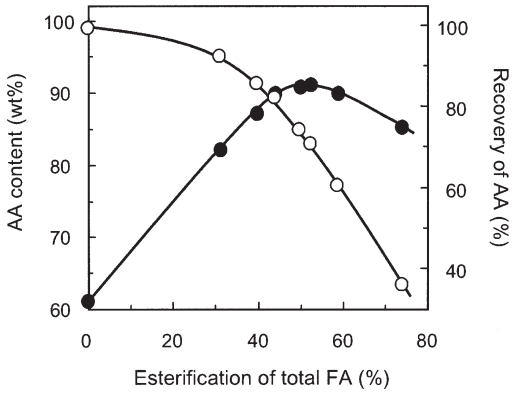

FIG. 3. Selective esterification of FFA-Urea using Lipase-PS. A mixture of 4 g FFA-Urea/LauOH (1:2, mol/mol) and 1 g water was stirred at 30°C for 16 h using 0, 10, 20, 30, 50, 60, 90, and 180 U/g-mixture of lipase. (●) Content of AA in the FFA fraction; (○) recovery of AA based on the content in FFA-Urea. See Figures 1 and 2 for abbreviations.

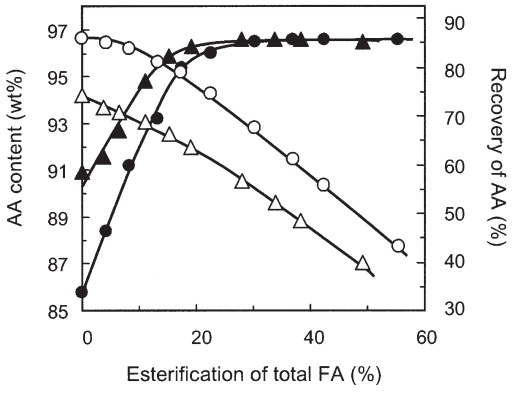

FIG. 4. Enrichment of AA in FFA-AA86 and FFA-AA91 by the second selective esterification using Lipase-PS. A mixture of 4 g FFA/LauOH (1:2, mol/mol) and 1 g water was stirred at 30°C for 16 h using 0, 2, 4, 8, 16, 25, 50, 75, 100, and 150 U/g-mixture of lipase. Circles, reaction with FFA-AA86; triangles, FFA-AA91. Closed symbols, content of AA in the FFA fraction; open symbols, recovery of AA based on the content in FFA-Urea. See Figures 1 and 2 for abbreviations.

different amounts of Lipase-PS. The content of AA in the FFA fraction increased from 61 to 91 wt% at 53% esterification and decreased at >53% esterification (Fig. 3). The recovery of AA was gradually decreased with an increasing degree of esterification, and was 71% based on the content in FFA-Urea when the degree of esterification reached 53%. This degree of esterification was attained using 60 U/g-mixture of the lipase.

Repeated selective esterification. A single selective esterification increased the purity of AA to 91%, with a 71% recovery based on the initial content of FFA-Urea. To further increase the purity in a good yield, FFA were recovered from the reaction mixture and used in a repeated esterification.

If the first esterification were stopped before the content of AA reached a maximum value and the unesterified FFA were allowed to react again, the recovery of AA could increase. Hence, two different FFA mixtures were prepared for the second selective esterification: A mixture containing FFA-Urea/LauOH (1:2, mol/mol), 20 wt% water, and 20 or 60 U/g-mixture of Lipase-PS was incubated at 30°C for 16 h. The contents of AA in the FFA fractions were 86 and 91 wt%, and the recoveries of AA were 86 and 74%, respectively. The FFA fractions were named FFA-AA86 and -AA91, respectively.

FFA-AA86 and -AA91 were esterified at 30°C for 16 h with 2 molar equivalents of LauOH using different amounts of Lipase-PS (Fig. 4). Regardless of the substrates, the AA content increased with an increasing degree of esterification and reached an approximately constant value (97 wt%) at >30% of esterification. The recovery of AA, based on the initial content in FFA-Urea, was higher when FFA-AA86 was used as a substrate than when FFA-AA91 was used.

Based on these results, the first selective esterification of FFA-Urea was determined so that the content of AA in the FFA fraction became *ca*. 85 wt% (degree of esterification, *ca*. 40%): A mixture of FFA-Urea/LauOH (1:2 mol/mol), 20 wt% water, and 20 U/g-mixture of Lipase-PS was stirred at 30°C for 16 h. The unesterified FFA mixture was named FFA-Est1. The second selective esterification was decided so that the degree of esterification became *ca*. 30%: A mixture of FFA-Est1/LauOH (1:2 mol/mol), 20 wt% water, and 50 U/g-mixture of Lipase-PS was stirred at 30°C for 16 h.

TABLE 2
Purification of AA (20:4) from TGA40 Oil

Step	Weight (g)	FA composition (wt%)[a]									Recovery of AA (%)
		16:0	18:0	18:1	18:2	18:3	20:3	20:4	22:0	24:0	
TGA40 oil[b]	100	9.9	6.9	6.6	9.2	2.6	3.5	44.4	3.4	9.3	100
Hydrolysis (FFA-Hyd)[c]	89.6	9.5	6.7	6.6	9.0	2.5	3.5	45.3	3.4	9.3	95.3
Urea adduct (FFA-Urea)	61.7	5.5	1.0	8.1	11.8	3.3	4.7	60.8	ND[d]	ND	88.5
Esterification (FFA-Est1)[e]	37.5	1.5	0.4	3.4	2.9	0.8	1.8	85.7	ND	ND	69.6
Esterification (FFA-Est2)[f]	21.7	0.2	ND	0.3	0.3	ND	ND	96.6	ND	ND	48.9

[a]FA composition in the FFA fraction.
[b]FA composition in TGA40 oil (Suntory Ltd., Osaka, Japan).
[c]A mixture of 100 g TGA40 oil, 200 mL water, and 1200 U/g-mixture of *Alcaligenes* sp. lipase was agitated at 40°C and 250 rpm for 48 h.
[d]ND, not detected (<0.1 wt%).
[e]A mixture of FFA-Urea/LauOH (1:2, mol/mol), 20 wt% water, and 20 U/g-mixture of Lipase-PS was stirred at 30°C for 16 h.
[f]A mixture of FFA-Est1/LauOH (1:2, mol/mol), 20 wt% water, and 50 U/g-mixture of Lipase-PS was stirred at 30°C for 16 h. See Table 1 for abbreviations.

Purification of AA by a series of procedures. Purification of AA was performed using 100 g TGA40 oil as a starting material (Table 2). The oil was hydrolyzed at 40°C for 48 h with *Alcaligenes* sp. lipase, and FFA-Hyd was recovered from the hydrolysate by extraction with *n*-hexane (the recovery of AA, 95%). Urea adduct fractionation of FFA-Hyd increased the content of AA from 45 to 61 wt% with an 89% recovery based on the initial content of TGA40 oil. The resulting FFA mixture (FFA-Urea) was then subjected to repeated selective esterification, and unesterified FFA (FFA-Est1) were recovered from the reaction mixture by extraction with *n*-hexane. The purified preparation (22 g; FFA-Est2) contained negligible amounts of GLA, DGLA, and stearic acid, and AA was purified to 97 wt% with a 49% recovery based on the initial content in TGA40 oil. The contents of LauOH and FA lauryl esters in FFA-Est2 were 0.6 and <0.1 wt%, respectively. The content of AA was higher than that in the preparation obtained by our previous process with repeated selective esterification using *C. rugosa* lipase (81 wt% AA) (13), and the recovery was the same as that by the previous process (48%) (13).

In conclusion, this process involving selective esterification with LauOH using Lipase-PS was very effective for producing highly purified AA from TGA40 oil. Furthermore, this process required only simple and conventional equipment and could handle a large amount of material. Hence, this process may be applied for the industrial purification of AA.

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