Characterization of Primary Fatty Amides Produced by Lipase-Catalyzed Amidation of Hydroxylated Fatty Acids

William E. Levinson[a], Tsung Min Kuo[a,*], and David Weisleder[b]

[a]Microbial Genomics and Bioprocessing and [b]New Crops and Processing Technology Research Units, NCAUR, ARS, USDA, Peoria, Illinois 61604

ABSTRACT: A novel hydroxylated primary fatty amide was produced from lesquerolic acid by direct amidation with ammonia catalyzed by immobilized *Pseudozyma (Candida) antarctica* lipase B (Novozym 435) in organic solvent. The amides of ricinoleic acid and oleic acid were also produced for comparison. The hydroxy FA were transformed at comparable rates to that of oleic acid. The rate of amide formation was greater for the longer-chain lesquerolic acid than for ricinoleic acid. All products exhibited characteristic primary-amide mass spectrum peaks with a base peak at *m/z* 59 and a strong ion fragment at *m/z* 72. Other peaks present are consistent with cleavage on either side of the hydroxyl position. The mass spectra, together with ^{1}H and ^{13}C NMR data, suggest that the products of lipase-catalyzed direct amidation of ricinoleic acid and lesquerolic acid are 12-hydroxy-9(*Z*)-octadecenamide and 14-hydroxy-11(*Z*)-eicosenamide, respectively.

Paper no. J11020 in *JAOCS 82,* 501–504 (July 2005).

KEY WORDS: Hydroxy fatty acids, hydroxy fatty amides, lipase-catalyzed amidation, structural analysis, transformation.

Hydroxy FA have a number of industrial applications and are useful as chemical intermediates in synthesis reactions (1–3). Hydroxy FA derived from plants include ricinoleic acid [12-hydroxy-9(*Z*)-octadecenoic acid] (RA), the primary feedstock for the production of nylon-11 (4), and lesquerolic acid [14-hydroxy-11(*Z*)-eicosenoic acid] (LQA). Fatty amides are used in a wide variety of industrial and commercial applications (4). Primary unsubstituted fatty amides, such as oleamide and erucamide, are used as slip and anti-blocking agents in plastics processing (5). Production of hydroxylated primary amides may result in compounds with useful property differences, such as variations in m.p. or solubility. Also, the hydroxyl group could be subjected to additional chemical reactions to change the functionality of the compounds. Industrial preparation of primary amides is energy intensive (4), and so enzymatic synthesis has been investigated as a possibly less costly means of production.

The primary method for enzyme-catalyzed synthesis of fatty amides is the use of lipase (EC 3.1.1.3) in organic solvent. In early studies, secondary amides were produced by reacting FA esters or TG with alkylamines (6,7). Work then progressed through the lipase-catalyzed ammoniolysis of FA esters with ammonia for the production of primary fatty amides (8–13) to the direct amidation of FA with ammonia (14).

Here we report on the characterization of ricinoleamide and a novel hydroxylated primary fatty amide, lesquerolamide, produced by the lipase-catalyzed direct amidation of the corresponding FA.

MATERIALS AND METHODS

Oleic acid [9(*Z*)-octadecenoic acid] (OA) and RA were from Nu-Chek-Prep, Inc. (Elysian, MN). LQA was produced in the laboratory from *Lesquerella fendleri* oil by basic saponification. Immobilized *Candida antarctica* lipase B (Novozym 435; a product of Novozymes A/S, Bagsvaerd, Denmark), 2-methyl-2-butanol (*tert*-amyl alcohol, TAA), and ammonium carbamate were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were used without further purification.

Amidation reactions consisted of 15 mL of TAA containing 100 mM FA, 100 mM ammonium carbamate, and 150 mg (10 mg/mL) immobilized enzyme. Reaction mixtures were equilibrated overnight at 25, 35, 45, and 55°C prior to the addition of enzyme to start the reaction. Reactions were performed in triplicate in septum-sealed 16 × 125 mm screw-capped test tubes. Mixing was by a Labquake test-tube rotator (Barnstead Intl., Dubuque, IA). Samples were taken through the septa by syringe. Reactions were stopped by removal of the immobilized enzyme by filtration. For enzyme recycle reactions, the catalyst was rinsed with ethyl acetate and allowed to dry prior to addition to new, equilibrated, reaction mixtures.

OA, RA, and LQA transformation was determined by GC with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm film thickness) and FID detector. FA were converted to methyl esters with diazomethane prior to analysis. Analytical conditions were as follows: injector, 200°C; detector, 290°C; initial oven temperature, 200°C for 1 min, ramping to 270°C at 10°C/min, and holding for 10 min.

The reaction solvent was removed from the filtered reaction mixture by vacuum evaporation, and the products were purified by crystallization. The purified RA and LQA products, not subjected to diazomethane treatment, were analyzed by electron impact GC–MS at 70 eV under similar conditions to those described above.

Proton, carbon, distortionless enhancement by polarization transfer, homonuclear correlation spectroscopy, and heteronu-

*To whom correspondence should be addressed at Microbial Genomics and Bioprocessing Research Unit, USDA-ARS-NCAUR, 1815 N. University St., Peoria, IL 61604. E-mail: kuotm@ncaur.usda.gov

clear single-quantum coherence NMR spectra were obtained with a Bruker Avance 500 spectrometer (Billerica, MA) equipped with a 5-mm broadband inverse probe (^{13}C NMR, 125 MHz; ^{1}H NMR, 500 MHz). The solvent was deuterated chloroform, which served as the internal standard.

^{1}H NMR of proposed lesquerolamide [14-hydroxy-11(*Z*)-eicosenamide] showed the following resonance signals: olefinic protons –C*H*=C*H*– at 5.58 (1H, *m*) and 5.44 (1H, *m*); a hydroxyl proton at 3.63 (1H, *m*); protons α to carbonyl and adjacent to –C–O– at 2.23 (4H, *t*, *J* = 7.6 Hz); protons β to carbonyl at 1.65 (2H, *m*); methylene groups –CH_2– adjacent to –C*H*=C*H*– at 2.07 (2H, *m*), adjacent to –C–OH at 1.49 (2H, *m*), and the others at 1.30–1.40 (20H, *m*); and a terminal –CH_3 at 0.91 ppm (3H, *t*, *J* = 6.7 Hz). The ^{13}C NMR spectra indicated a total of 20 carbons and confirmed the presence of –*C*=O at C_1 (175.7 ppm); one –*C*–OH at 71.5 (C_{14}); two olefinic carbons –*C*H=*C*H– at 133.3 and 125.3 for C_{11} and C_{12}, respectively; 15 –*C*H_2–, ranging from 22.6 to 36.9 ppm ($C_{2–10,13,15–19}$); and one –*C*H_3 at 14.1 ppm (C_{20}). The double bond was determined to be a *cis* configuration because of the ^{13}C NMR chemical shift at C_{10} of 27.3 ppm (15). NMR analysis revealed that resonance signals and assignments for the proposed ricinoleamide (data not shown) were comparably identical to those of lesquerolamide except for the latter having two additional methylene groups.

RESULTS AND DISCUSSION

Ester ammoniolysis and the direct amidation of FA with ammonia catalyzed by *Pseudozyma (Candida) antarctica* lipase B (CALB, also know as Novozym 435 when immobilized) have been carried out in a number of solvents (11,12,14). For the direct amidation of OA to oleamide, Slotema *et al*. (16) determined that TAA provided the best combination of initial reaction rate and equilibrium conversion of four solvents tested. Addition of ammonia in the solid form (ammonium carbamate or ammonium bicarbonate) may avoid high concentrations of ammonia and the possible precipitation of FA ammonia salts, which can reduce the initial reaction rate (14). Ammonium carbamate is preferred as an ammonia source (over ammonia bicarbonate) because it does not form water as it dissolves, and an increase in the initial water concentration of the reaction mix results in lower equilibrium transformation and initial reaction rates (16). Given these results, we selected TAA as solvent and ammonium carbamate as ammonia source for our work.

Transformation reactions were performed at 25, 35, 45, and 55°C. The ammonia/FA molar ratio for these experiments was 2:1 (100 mM ammonium carbamate giving 200 mM ammonia). This concentration of ammonium carbamate was completely soluble in TAA at 55°C, but not at the lower temperatures. Transformations were monitored as the utilization of the FA substrate and expressed as the percentage of substrate removed from the reaction. For each substrate there was only one product peak evident on GC chromatograms, and it was assumed that all substrate used was transformed into the amide products. Figure 1 shows the transformation of RA and LQA at 25 and 55°C. Apparently, CALB acted on the longer-chain LQA more rapidly than RA for direct amidation. The reason for the reaction preference is not known. RA required 7 d to approach completion at 25°C, whereas LQA transformation approached completion at 3 d at this temperature. At 55°C, the transformation of both substrates approached completion within 24 h. OA transformation was similar to RA. Under these conditions, the end point transformation (at 7 d and 55°C) of LQA and RA was 95.2 and 95.3%, respectively.

The stability of the activity of the biocatalyst in multiple consecutive reactions was tested by recycling the enzyme to new reaction mixtures with LQA as substrate. A series of five reactions was performed with enzyme recycle at 3- or 4-d intervals. There was no loss of activity seen at 24 h, with all reactions transforming better than 95% of the substrate at this time. Given that at 55°C the substrates are soluble at the concentrations used and

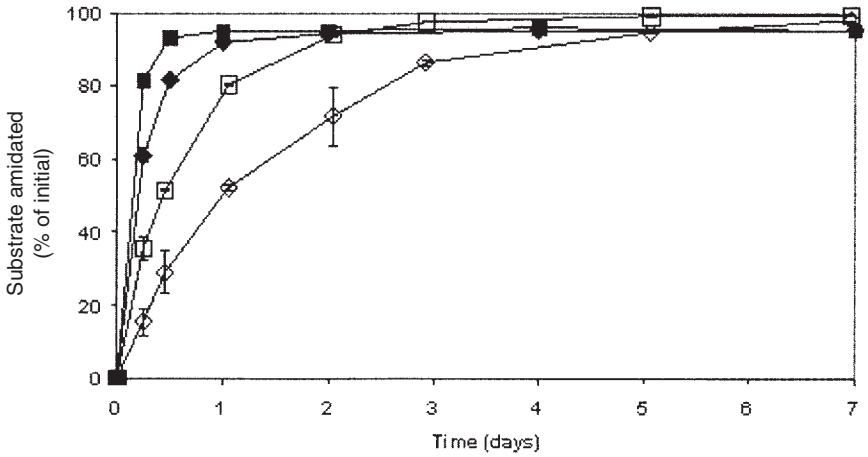

FIG. 1. FA transformation at 25 (□, ◇) and 55°C (■, ◆). Lesquerolic acid [14-hydroxy-11(*Z*)-eicosenoic acid], (□, ■); ricinoleic acid [12-hydroxy-9(*Z*)-octadecenoic acid] (◇,◆). Data points are the average of three replicates ± 1 SD.

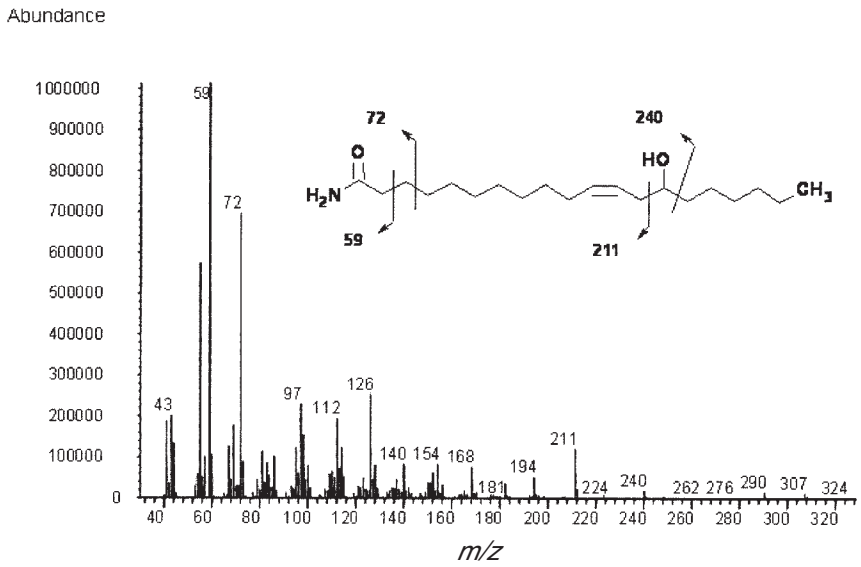

FIG. 2. Mass spectrum of lesquerolic acid transformation product.

that no other reactants are required, the batch scale-up of this system for industrial production seems feasible if adequate mixing is provided. The ability to recycle the biocatalyst to new reactions would be an important economic consideration.

Purified products from the OA, RA, and LQA reactions were analyzed by GC–MS. Authentic oleamide (Sigma-Aldrich) and the OA reaction product were both identified by a mass spectral library (17) as 9(*Z*)-octadecenamide (oleamide). The spectrum of the LQA product (Fig. 2) shows strong characteristic amide peaks at *m/z* 59 and 72, resulting from McLafferty rearrangement and γ-cleavage, respectively (18). The putative molecular ion is not present (lesquerolamide, FW 325.5); however, there is a signal at $(M - 18)^+$. Peaks indicating the position of the hydroxyl group occur at *m/z* 211 and 240. These fragments are likely the result of cleavage on either side of an oxygen radical (19). The spectrum of the RA product (not shown) was similar to that of the LQA product, showing the characteristic amide peaks, an $(M - 18)^+$ signal at *m/z* 279, and peaks at *m/z* 183 and 212 indicating the position of the hydroxyl group. The NMR data supported the analysis of the chemical structures suggested by the mass spectra; therefore, the new compound derived from LQA was 14-hydroxy-11(*Z*)-eicosenamide.

ACKNOWLEDGMENTS

The authors wish to thank Jim Nicholson and Dr. Neil Price for mass spectral analysis. Names are necessary to report factually on available data; however, the USDA neither guarantees not warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

REFERENCES

1. Hayes, D.G., The Catalytic Activity of Lipases Toward Hydroxy Fatty Acids: A Review, *J. Am. Oil Chem. Soc. 73*:543–549 (1996).
2. Biermann, U., W. Friedt, S. Lang, W. Lühs, G. Machmüller, J.O. Metzger, M. Rüsch gen. Klaas, H.J. Schäfer, and M.P. Schneider, New Syntheses with Oils and Fats as Renewable Raw Materials for the Chemical Industry, *Angew. Chem. Int. Edit. 39*:2206—2224 (2000).
3. Hou, C.T., T.M. Kuo, and A.C. Lanser, Value-Added Products Through Bioprocessing: New Hydroxy Fatty Acids, *inform 13*:307–316 (2002).
4. Opsahl, R., Amides, Fatty Acid, in *Kirk-Othmer Encyclopedia of Chemical Technology*, edited by. J.I. Kroschwitz and M. Howe-Grant, Vol. 2, 4th edn., John Wiley & Sons, New York, 1992, pp. 346–356.
5. Zweifel, H. (ed.), *Plastics Additives Handbook*, Hanser Gardner Publications, Cincinnati, 2001, pp. 511–607.
6. Bistline, R.G., A. Bilyk, and S.H. Feairheller, Lipase Catalyzed Formation of Fatty Amides, *J. Am. Oil Chem. Soc. 68*:95–98 (1991).
7. Gotor, V., R. Brieva, C. Gonzalez, and F. Rebolledo, Enzymatic Aminolysis and Transamidation Reactions, *Tetrahedron 47*:9207–9214 (1991).
8. de Zoete, M.C., A.C. Kock-van Dalen, F. van Rantwijk, and R.A. Sheldon, Ester Ammoniolysis: A New Enzymatic Reaction, *J. Chem. Soc., Chem. Commun. 24*:1831–1832 (1993).
9. Garcia, M.J., F. Rebolledo, and V. Gotor, Chemoenzymatic Aminolysis and Ammonolysis of β-Ketoesters, *Tetrahedron Lett. 34*:6141–6142 (1993).
10. de Zoete, M.C., A.C. Kock-van Dalen, F. van Rantwijk, and R.A. Sheldon, A New Enzymatic Reaction: Enzyme Catalyzed Ammoniolysis of Carboxylic Esters, *Biocatalysis 10*:307–316 (1994).
11. Garcia, M.J., F. Rebolledo, and V. Gotor, Lipase-Catalyzed Aminolysis and Ammonolysis of β-Ketoesters. Synthesis of Optically Active β-Ketoamides, *Tetrahedron 50*:6935–6940 (1994).
12. de Zoete, M.C., A.C. Kock-van Dalen, F. van Rantwijk, and R.A. Sheldon, Lipase-Catalysed Ammoniolysis of Lipids. A Facile Synthesis of Fatty Acid Amides, *J. Mol. Catal. B–Enzym. 1*:109–113 (1996).
13. de Zoete, M.C., A.C. Kock-van Dalen, F. van Rantwijk, and R. A. Sheldon, A New Enzymatic One-Pot Procedure for the Synthesis of Carboxylic Amides from Carboxylic Acids, *Ibid. 2*:19–25 (1996).

14. Litjens, M.J.J., A.J.J. Straathof, J.A. Jongejan, and J.J. Heijnen, Exploration of Lipase-Catalyzed Direct Amidation of Free Carboxylic Acids with Ammonia in Organic Solvents, *Tetrahedron 55*:12411–12418 (1999).
15. Lee, K.J., and A.K.L. Cheng, Confirmation of the Carbon Chemical Shifts of Ethylenic Carbon Atoms in Methyl Ricinoleate and Methyl Ricinelaidate, *Nat. Prod. Lett. 3*:65–69 (1993).
16. Slotema, W.F., G. Sandoval, D. Guieysse, A.J. Straathof, and A. Marty, Economically Pertinent Continuous Amide Formation by Direct Lipase-Catalyzed Amidation with Ammonia, *Biotechnol. Bioeng. 82*:664–669 (2003).
17. McLafferty, F.W., *Wiley Registry of Mass Spectral Data*, 7th edn., John Wiley & Sons, Hoboken, NJ, 2003.
18. Budzikiewicz, H., C. Djerassi, and D.H. Williams, *Mass Spectrometry of Organic Compounds*, Holden-Day, San Francisco, 1967, pp. 336–353.
19. Murphy, R.C., *Mass Spectrometry of Lipids*: *Vol. 7*, *Handbook of Lipid Research*, edited by F. Snyder, Plenum Press, New York, 1993, pp. 99–102.

[Received December 28, 2004; accepted May 17, 2005]