BIOORGANIC CHEMISTRY 15, 141–151 (1987)

Total Synthesis of Human Plasma Apolipoprotein C-II[1]

AMBIKAIPAKAN BALASUBRAMANIAM,[2] YASH M. CHOPRA, MARK T. YATES, AND RICHARD L. JACKSON

Departments of Surgery, Medicine, and Pharmacology and Biophysics, University of Cincinnati Medical Center, Cincinnati, Ohio 45267, and Merrell Dow Research Institute, 2110 East Galbraith Road, Cincinnati, Ohio 45215

Received September 12, 1986

Apolipoprotein C-II (apoC-II), a protein of 79 amino acid residues present in very low density lipoproteins, enhances the lipoprotein lipase (LpL)-catalyzed hydrolysis of triacylglycerols transported in plasma triglyceride-rich lipoproteins. To elucidate the structure–activity relationship of this activator protein, the complete amino acid sequence of apoC-II has been synthesized by the solid-phase method with Boc-amino acid derivatives and phenylacetamidomethyl resin. The crude peptide was purified to homogeneity in 10% yield by a combination of ion-exchange and preparative high-performance liquid chromatography (HPLC). The purified peptide had the expected amino-terminal sequence and amino acid composition. Synthetic and native apoC-II were indistinguishable by cochromatography on analytical HPLC, peptide mapping of tryptic digest, radioimmunoassay, and activation of LpL with both artificial and lipoprotein substrates.

Chylomicrons and very low density lipoproteins (VLDL)[3] represent the major carriers of triacylglycerols in the circulation. These triglyceride-rich lipoproteins are acted upon by lipoprotein lipase (LpL) at the capillary endothelium, yielding free fatty acids and monoglycerides (*1*). For maximal activity, LpL requires an activator protein, termed apolipoprotein C-II (apoC-II) (*2*), which is associated with the lipoprotein particle. ApoC-II is a single polypeptide chain of 79 amino acid residues (*3*) of known sequence; the DNA sequence of apoC-II has also been determined (*4–7*). Structure–activity studies have shown that the minimal amino acid sequence of apoC-II required for activation of LpL is dependent on the composition and physical state of the lipid substrate (*8–11*). For short-chain fatty acyl lipids (≤8 carbon atoms), the minimal sequence resides between residues 56 and 79; however, for fatty acyl lipid substrates of 16 carbon atoms or greater, a lipid-binding region contained within residues 1–55 of apoC-II is required for maximal activation. We recently showed that the attachment of a *N*-α-palmitoyl

[1] This research was supported in part by U.S. Public Health Grants HL-22619 and HL-23019. We gratefully acknowledge the assistance of Mrs. Rita Hoeper in preparing the manuscript for publication.

[2] To whom correspondence should be addressed at Department of Surgery, University of Cincinnati Medical Center, Cincinnati, Ohio 45267.

[3] Abbreviations: VLDL, very low density lipoproteins; apoC-II, apolipoprotein C-II; DCC, dicyclohexylcarbodiimide; DIPEA, diisopropylethylamine; HOBT, 1-hydroxybenzotriazole; LpL, lipoprotein lipase; PAM, phenylacetamidomethyl; TFA, trifluoroacetic acid. Amino acid derivatives and peptides are those recommended by the IUPAC–IUB commission.

0045-2068/87 $3.00

Boc-Thr(Bzl)-Gln-Gln-Pro-Gln-Gln-Asp(OBzl)-Glu(OBzl)-Met-Pro-Ser(Bzl)-Pro-Thr(Bzl)-Phe-
-Leu-Thr(Bzl)-Gln-Val-Lys(ClZ)-Glu(OBzl)-Ser(Bzl)-Leu-Ser(Bzl)-Ser(Bzl)-Tyr(BrZ)-Trp
(CHO)-Glu(OBzl)-Ser(Bzl)-Ala-Lys(ClZ)-Thr(Bzl)-Ala-Ala-Gln-Asn-Leu-Tyr(BrZ)-Glu(OBzl)-
Lys(ClZ)-Thr(Bzl)-Tyr(BrZ)-Leu-Pro-Ala-Val-Asp(OBzl)-Glu(OBzl)-Lys(ClZ)-Leu-Arg(Tos)-
Asp(OBzl)-Leu-Tyr(BrZ)-Ser(Bzl)-Lys(ClZ)-Ser(Bzl)-Thr(Bzl)-Ala-Ala-Met-Ser(Bzl)-Thr
(Bzl)-Tyr(BrZ)-Thr(Bzl)-Gly-Ile-Phe-Thr(Bzl)-Asp(OBzl)-Gln-Val-Leu-Ser(Bzl)-Val-Leu-
Lys(ClZ)-Gly-Glu(OBzl)-Glu(OBzl)-PAM-RESIN,

FIG. 1. Protection scheme for the solid-phase synthesis of apoC-II.

moiety to residues 56–79 mimics the lipid-binding region of apoC-II (*12*). As part of a continuing effort to understand the relationship between the structure and function of apoC-II, we now report the total synthesis of apoC-II.

MATERIALS AND METHODS

Materials. Boc-amino acid derivatives and aminomethyl resin (Peninsula Laboratories), 4-(bromomethyl)phenylacetic acid phenacyl ester (RSA Corp.), sequential grade trifluoroacetic acid and diisopropylethylamine (Chemical Dynamic Corp.), and glass-distilled solvents (Burdick and Jackson) were used without further purification. Tri[1-^{14}C]oleoylglycerol (90 mCi/mmol) and tri[9,10-^{3}H-(N)] oleoylglycerol (121 Ci/mmol) were purchased from New England Nuclear. Heparin (porcine mucosal, 169 units/mg), fatty acid free bovine serum albumin (BSA), and trioleoylglycerol were purchased from Sigma Chemical Company. Native apoC-II was isolated from triglyceride-rich lipoproteins of density <1.006 g/ml from fasting subjects with familial endogenous hypertriglyceridemia with chylomicronemia (type V hypertriglyceridemia) as described previously (*13*); apoC-II was further purified by HPLC as described below. LpL was isolated from human postheparin plasma as described previously (*11*). ApoC-II deficient VLDL were isolated by ultracentrifugation from plasma of a patient (A.G.) with deficiency of the activator protein (*14*). Tri[^{3}H]oleoylglycerol was incorporated into VLDL according to the procedure of Fielding (*15*) as described previously (*16*).

Synthesis of apoC-II. Boc-Glu(OBzl)-PAM-resin (2 g, 0.48 mmol), synthesized according to the procedure of Mitchell *et al.* (*17*), was placed in the reaction vessel of the peptide synthesizer (Vega Model 50 with AIM 65 controller), and the synthesis was performed coupling the protected amino acid derivatives sequentially as shown in Fig. 1 and Table 1. The preformed symmetrical anhydrides and HOBT esters were prepared just before coupling by the following methods: (i) A solution of protected amino acid derivative (3.84 mmol, 8 equivalents) in CH_2Cl_2 (5 ml) was cooled to 0°C, and DCC (1.92 mmol) in CH_2Cl_2 (25 ml) was added. The mixture was stirred at 0°C for 10 min. The precipitate of dicyclohexylurea was removed by filtration and the preformed symmetrical anhydride was added immediately to the reaction vessel. (ii) A solution of HOBT (1.92 mmol) in DMF (10 ml) was cooled to 0°C, and DCC (1.92 mM) in CH_2Cl_2 (5 ml) and protected amino acid derivative (1.92 mmol) in CH_2Cl_2 (20 ml) were added. After it was stirred at 0°C for

TABLE 1

STEPS IN THE SOLID-PHASE SYNTHESIS OF APOLIPOPROTEIN C-II

	Step	Procedure
1	Washing	CH_2Cl_2 (3 × 1 min)
2	Deblocking	50% TFA–CH_2Cl_2 (1 × 2 min + 1 × 28 min)
3	Washing	CH_2Cl_2 (5 × 1 min)
4	Neutralization	5% DIPEA–CH_2Cl_2 (2 × 3 min)
5	Washing	CH_2Cl_2 (4 × 1 min)
6	Coupling	4 eq. preformed symmetrical anhydride (1 × 120 min)
7	Neutralization	5% DIPEA–CH_2Cl_2 (1 × 2 min)
8	Washing	CH_2Cl_2 (4 × 1 min)
9	Coupling	4 eq. preformed HOBT ester (1 × 120 min)
10	Washing	DMF (2 × 1 min)
11	Repeat	Steps 7 and 8
12	Monitoring	Qualitative ninhydrin test

10 min, the mixture was added to the reaction vessel. Boc-Asn, Boc-Gln, and Boc-Arg (Tos) were coupled twice by preformed HOBT esters. At each step in the synthesis, a qualitative ninhydrin test was performed at the end of the second coupling and, if required, the coupling was repeated for a third time. At the end of the synthesis, the peptide resin was dried *in vacuo* at room temperature for 18 h.

Peptide was cleaved from the resin with HF (*18*). Peptide resin (0.15 mmol), dimethylsulfide (3.2 ml), *p*-cresol (0.5 g), and *p*-thiocresol (0.25 g) were placed in the reaction vessel of the HF apparatus; HF was collected to a final volume of 5 ml at −76°C. The mixture was stirred at 0°C for 2 h. HF was removed and the residue was washed by decantation with diethyl ether (2 × 20 ml). The peptide residue was dried in a stream of N_2 and was again treated with liquid HF (5 ml) containing *p*-cresol (0.5 g) and *p*-thiocresol (0.25 g) for 1 h at 0°C as described above. After repeated washings with diethyl ether, the crude peptide product was extracted (3 × 5 ml) with TFA containing 1% 2-mercaptoethanol. The extracts were combined and the peptides were precipitated with diethyl ether. The precipitate was collected by centrifugation and dried first in a stream of N_2 and then *in vacuo* at room temperature for 18 h.

Purification of synthetic ApoC-II. Crude peptide (120 mg) was dissolved in 6 M guanidine–HCl, dialyzed (3500 MW cutoff tubing) extensively against 10 mM NH_4HCO_3, and lyophilized. Peptides (80 mg) were dissolved in a minimal volume of 6 M urea and the pH was adjusted to 8.0 with 1 M NaOH. The conductivity of the peptide solution was then adjusted to ≤0.5 mS by dilution with 6 M urea. The solution was then applied to a column (2.5 × 35 cm) of DEAE-Sephacel (Pharmacia) at 4°C, equilibrated with 10 mM Tris–HCl, pH 8.0, 6 M urea. After the peptide entered the resin it was eluted with a linear gradient (1 liter) of NaCl (0–0.125 M) in the equilibration buffer. The flow rate was 25 ml/h and 10-ml fractions were collected. Peptide was monitored by measuring the absorbance at 280 nm and those fractions corresponding to native apoC-II were pooled, dialyzed against 10 mM NH_4HCO_3, and lyophilized.

Synthetic apoC-II was further purified by HPLC (Milton Roy). Peptides (0.5–1.0 mg) in 100 μl of 6 M guanidine–HCl/0.1% TFA–H_2O were loaded onto a Beckman Ultrapore RPSC C-3 column (4.6 × 75 mm; particle size 5 μm; pore size 30 nm) and peptides were eluted with a 50-ml linear gradient of 0–60% CH_3CN in 0.1% TFA–H_2O over 50 min; flow rate was 1 ml/min. Fractions (0.5 ml) were collected and those peptides that eluted at the same retention time as native apoC-II were pooled, concentrated in a Speed-Vac centrifuge, diluted with water, and lyophilized.

Other methods. Peptide maps of native and synthetic apoC-II were determined by HPLC of tryptic digests. Peptides (100 μg) in 100 μl of 10 mM NH_4HCO_3 were incubated with 30% by weight L-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Millipore Corp.) for 5 h at room temperature. After boiling and lyophilization, the digests were dissolved 6 M guanidine–HCl/0.1% TFA–H_2O and subjected to HPLC as described above.

Isoelectric focusing was performed as described previously (*19*). Activation of LpL by native and synthetic apoC-II was determined both with a Triton X-100 emulsified trioleoylglycerol substrate (*20*) and apoC-II deficient VLDL (*16, 21*). The final concentration of triacylglycerol in the assay mixture was 1.5 mM. Enzyme incubations were performed in duplicate at 37°C in a total volume of 0.3 ml of 100 mM Tris–HCl, pH 8.6, 0.15 M NaCl, containing 10 mg fatty acid-free BSA and 20 μg heparin. Purified postheparin plasma LpL (20 μg) was added and, after 15 min, enzyme reactions were terminated and the initial rate of LpL catalysis was determined as described previously (*16, 20, 21*). LpL assays were performed with two different substrate preparations with identical results. The immunoreactivity of native and synthetic apoC-II was determined by radioimmunoassay with an antibody prepared against the native peptide (*22*). Amino acid compositions were determined on a Waters Pico-Tag system. Peptides attached to the resins were hydrolyzed at 130°C for 2 to 6 h with 1 : 1 mixture of 12 N HCl and propionic acid. Free peptides were hydrolyzed for 24 h at 110°C with 6 N HCl or 4 N methanesulfonic acid. Amino-terminal sequences were determined on a Model 470A gas-phase sequencer (Applied Biosystems).

RESULTS AND DISCUSSION

The synthesis of the entire 79 amino acid sequence of apoC-II was performed by the solid-phase method (*23*) and utilized N^{α}-t-Boc protection; the groups for side-chain protection are shown in Fig. 1. Several improvements were incorporated into the synthesis strategy to maximize the yield of peptide. The use of PAM resin, synthesized according to the procedure of Mitchell *et al.* (*17*), reduces the premature loss of the growing peptide chain and also chain termination by trifluoroacetylation (*24*). Each amino acid residue was coupled twice, first as the preformed symmetrical anhydride in CH_2Cl_2 and second as preformed HOBT esters in DMF. Alteration of the polarity of the coupling solvents has been shown to improve the coupling efficiency (*25*). For asparagine, glutamine, and arginine, couplings were effected both times by the preformed HOBT ester method to avoid

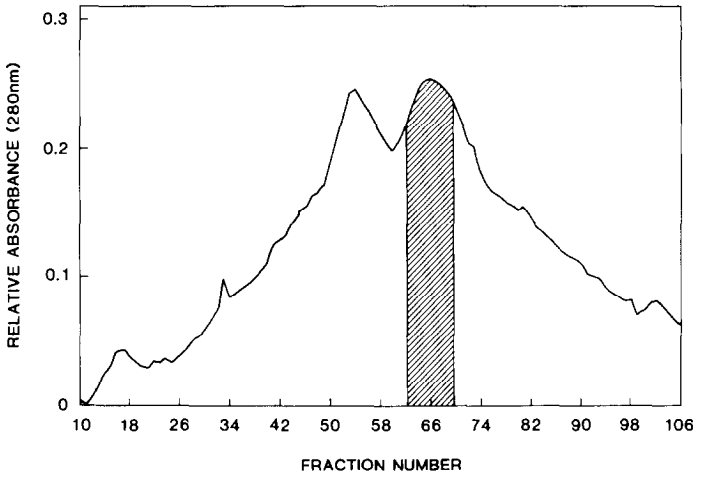

FIG. 2. DEAE-Sephacel chromatography of synthetic apoC-II obtained after HF cleavage. The column (2.5 × 35 cm) was eluted with a linear gradient of 0–0.125 M NaCl in 10 mM Tris–HCl, pH 8.0, 6 *M* urea; flow rate was 25 ml/h and 10-ml fractions were collected. (Shaded area corresponds to the elution profile of native apoC-II.)

side reactions (*25, 26*). At the end of the synthesis, peptides were released from the resin by the low–high HF method (*18*). This procedure eliminates most of the side reactions caused by carbocations, deformylates tryptophan, and reduces any oxidized methionine (*18*). Based on amino acid analysis of the HF-treated peptide resin, the amount of free peptide released from the resin was estimated to be 74% of the total.

Synthetic apoC-II was purified by sequential chromatography on DEAE-Sephacel and HPLC. The crude peptide showed multiple components when subjected to anion-exchange chromatography (Fig. 2). Those fractions corresponding to the elution profile for native apoC-II were pooled and subjected to reverse-phase HPLC (Fig. 3A). The absorbance profile at 220 nm showed a peak (43% acetonitrile) corresponding to that for the native peptide (Fig. 3B); these fractions were pooled and, if required, were rechromatographed. As discussed by Tam *et al.* (*27*), the broad spectrum of peptides observed in the crude mixture (Fig. 3A) results from the cleavage and side-chain deprotection steps, chemically damaged products, and chain-terminated fragments.

Synthetic apoC-II was compared with the native peptide by a number of analytical, chemical, immunochemical, and biological methods. The purified peptide was homogeneous by analytical HPLC and cochromatographed with native apoC-II (Fig. 4). Isoelectric focusing of synthetic apoC-II gave a single component with a p*I* value of 4.78 (Fig. 5). Furthermore, a mixture of native and synthetic apoC-II gave a single component (Fig. 5, lane C).

Table 2 gives the amino acid compositions of native and synthetic apoC-II. Within experimental error, the compositions were nearly identical. Automated Edman degradation of the amino-terminal 20 residues gave the expected amino

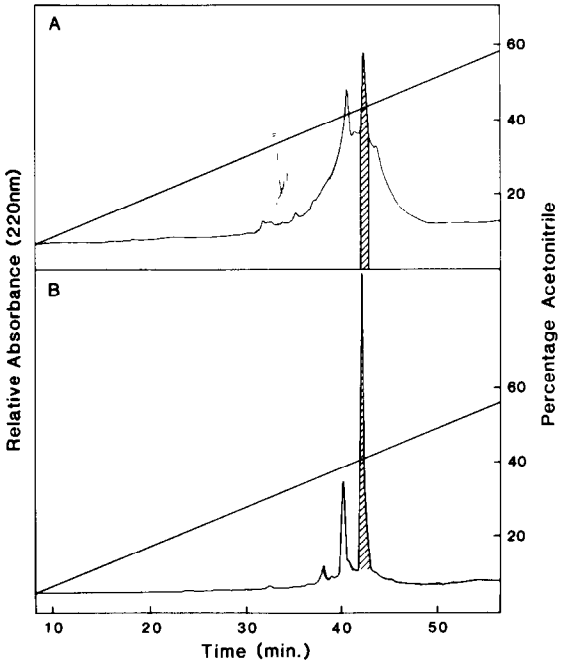

FIG. 3. HPLC of (A) synthetic and (B) native apoC-II obtained by ion-exchange chromatography (Fig. 2). Peptides were applied to a Beckman ultrapore RPSC column (4.6 × 75 mm, 5-μm particle size, 30-nm pore size) and eluted with a gradient of 0 to 60% CH_3CN/0.1% TFA–H_2O in 50 min; flow rate was 1 ml/min.

TABLE 2

AMINO ACID COMPOSITION OF NATIVE AND SYNTHETIC APOLIPOPROTEIN C-II

Amino acid	Native apoC-II	Synthetic apoC-II	Expected
Asx	4.28	4.33	5
Glx	13.99	13.45	14
Ser	8.04	8.56	9
Gly	2.20	2.53	2
Arg	1.00	1.00	1
Thr	7.56	7.70	9
Ala	5.74	5.50	6
Pro	4.54	4.50	6
Tyr	4.44	4.30	5
Val	3.66	4.00	4
Met	1.24	1.45	2
Ile	0.68	0.82	1
Leu	7.56	7.10	8
Phe	1.84	1.80	2
Lys	5.72	5.10	6
Trp	N.D.	0.90[a]	1

[a] Determined after hydrolysis with 4 N methanesulfonic acid.

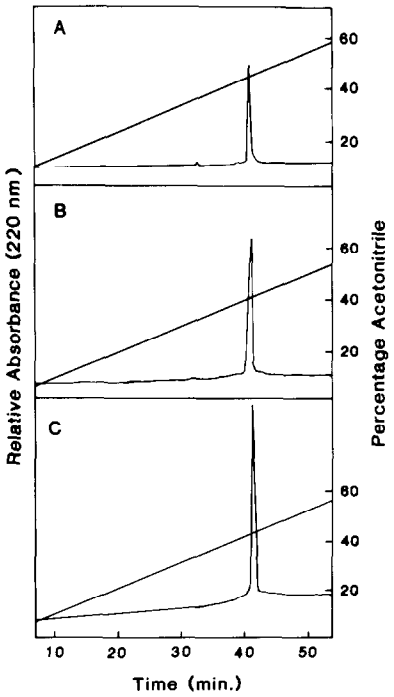

FIG. 4. Analytical HPLC of purified (A) synthetic apoC-II, (B) native apoC-II, and (C) a mixture of synthetic and native apoC-II. The experimental conditions are the same as in Fig. 3.

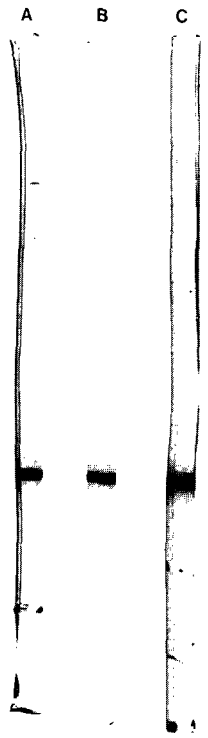

FIG. 5. Polyacrylamide isoelectric focusing of (A) synthetic apoC-II, (B) native apoC-II, and (C) mixture of native and synthetic apoC-II.

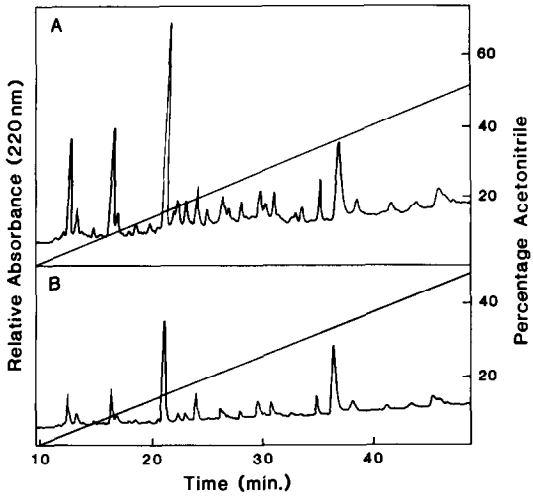

FIG. 6. Analytical HPLC of a tryptic digest of (A) synthetic and (B) native apoC-II. The experimental conditions are the same as in Fig. 3.

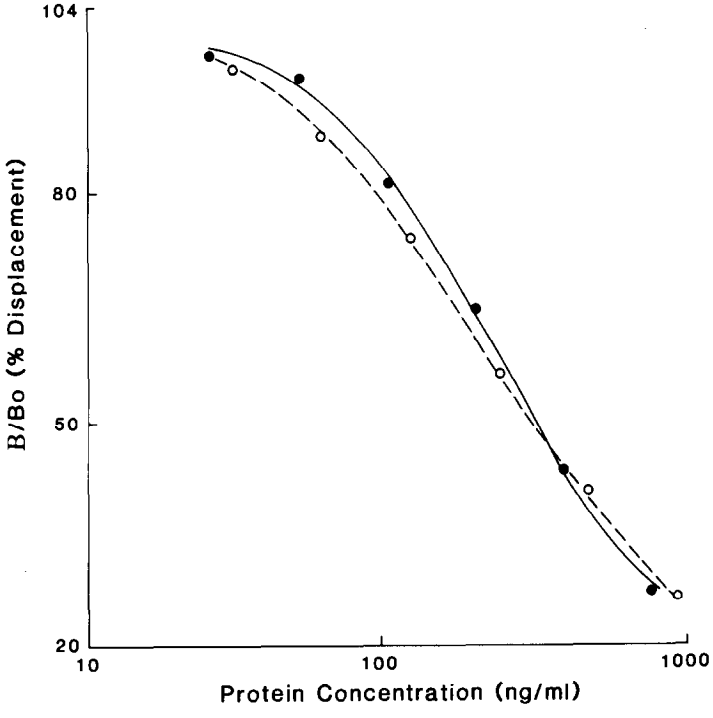

FIG. 7. Displacement of ^{125}I-labeled apoC-II by varying concentrations of synthetic (●) and native (○) apoC-II.

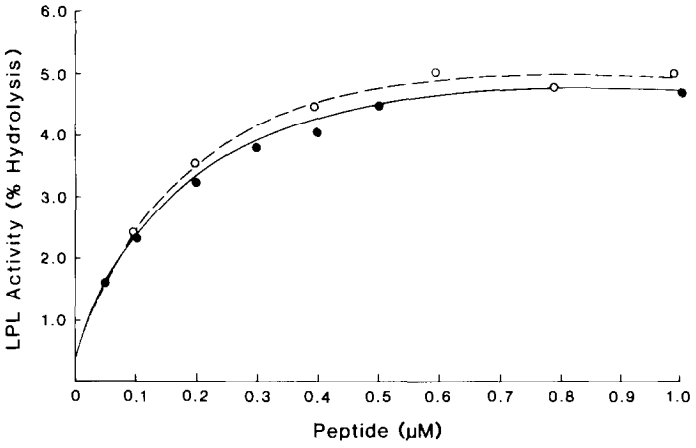

FIG. 8. Effect of synthetic (●) and native (○) apoC-II on the LpL-catalyzed hydrolysis of Triton X-100 emulsified tri[^{14}C]oleoylglycerol.

acid sequence with <10% background residues. The primary structure of the synthetic peptide was also compared with the native protein by tryptic-peptide mapping (Fig. 6). As determined by HPLC, native and synthetic apoC-II had nearly identical elution profiles of their tryptic peptides, providing further evidence for their similarity in sequence.

The immunoreactivity of synthetic and native apoC-II was compared by radioimmunoassay with an antiserum raised against native apoC-II (22). As is shown in Fig. 7, both peptides were nearly equivalent in displacing ^{125}I-labeled apoC-II bound to the antiserum. Synthetic and natural apoC-II were also equally

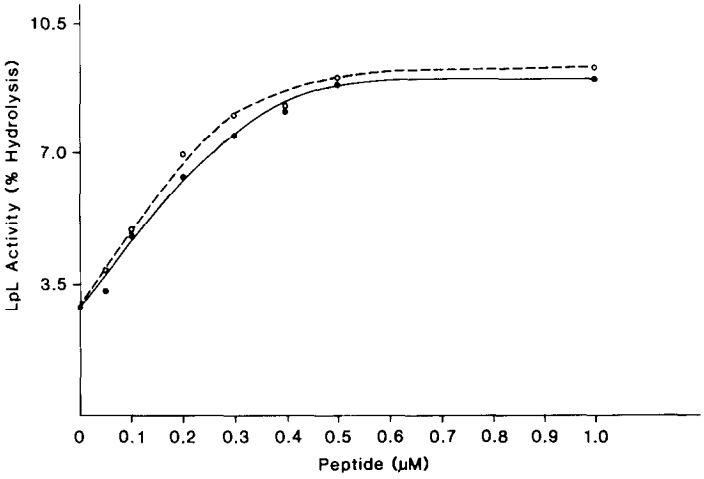

FIG. 9. Effect of synthetic (●) and native (○) apoC-II on the LpL-catalyzed hydrolysis of apoC-II deficient VLDL.

effective in enhancing the LpL-catalyzed hydrolysis of Triton X-100 emulsified tri[^{14}C]oleoylglycerol (Fig. 8) and apoC-II-deficient VLDL radiolabeled with tri-[^{3}H]oleoylglycerol (Fig. 9).

In summary, it is concluded that apoC-II synthesized by solid-phase methods is identical to the native macromolecule and provides the initial information required for structure–activity studies.

REFERENCES

1. JACKSON, R. L. (1983) Enzymes **16,** 141–181.
2. HAVEL, R. J., FIELDING, C. J., OLIVECRONA, T., SHORE, V. G., FIELDING, P. E., AND EGELRUD, T. (1973) *Biochemistry* **12,** 1828–1833.
3. HOSPATTANKAR, A. V., FAIRWELL, T., RONAN, R., AND BREWER, H. B. (1984) *J. Biol. Chem.* **259,** 318–322.
4. MYKLEBOST, O., WILLIAMSON, B., MARKHAM, A. F., MYKLEBOST, S. R., ROGERS, J., WOODS, D. E., AND HUMPHRIES, S. E. (1984) *J. Biol. Chem.* **259,** 4401–4404.
5. FOJO, S. S., LAW, S. W., AND BREWER, H. B., JR. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6354–6357.
6. JACKSON, C. L., BRUNS, G. A. P., AND BRESLOW, J. L. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2945–2949.
7. WEI, C-F., TSAO, Y-K., ROBBERSON, D. L., GOTTO, A. M., JR., BROWN, K., AND CHAN, L. (1985) *J. Biol. Chem.* **260,** 15211–15221.
8. FAIRWELL, T., HOSPATTANKAR, A. V., POWELL, G. M., OSBORNE, J. C., JR., AND KHAN, S. A. (1985) *in* Peptides: Structure and Function; Proceedings of the Ninth American Peptide Symposium (Deber, C. M., Hruby, V. J., and Kopple, K. D., Eds.), pp. 899–902, Pierce Chemical Company, Rockford, Illinois.
9. SMITH, L. C., VOYTA, J. C., CATAPANO, A. L., KINNUNEN, P. K. J., GOTTO, A. M., JR., AND SPARROW, J. T. (1980) *Ann. N.Y. Acad. Sci.* **348,** 213–221.
10. JACKSON, R. L., BALASUBRAMANIAM, A., MURPHY, R. F., AND DEMEL, R. A. (1986) *Biochim. Biophys. Acta* **875,** 203–210.
11. JACKSON, R. L., PONCE, E., MCLEAN, L. R., AND DEMEL, R. A. (1986) *Biochemistry* **25,** 1166–1170.
12. BALASUBRAMANIAM, A., RECHTIN, A., MCLEAN, L. R., AND JACKSON, R. L. (1986) *Biochem. Biophys. Res. Commun.* **137,** 1041–1048.
13. JACKSON, R. L., AND HOLDSWORTH, G. (1986) *in* Methods in Enzymology, Vol. 128, pp. 288–298, Academic Press, New York.
14. SAKU, K., CEDRES, C., MCDONALD, B., HYND, B. A., LIU, B. W., SRIVASTAVA, L. S., AND KASHYAP, M. L. (1984) *Amer. J. Med.* **77,** 457–462.
15. FIELDING, C. J. (1979) *Biochim. Biophys. Acta* **573,** 255–265.
16. MATSUOKA, N., SHIRAI, K., JOHNSON, J. D., KASHYAP, M. L., SRIVASTAVA, L. S., YAMAMURA, T., YAMAMOTO, A., SAITO, Y., KUMAGAI, A., AND JACKSON, R. L. (1981) *Metabolism* **30,** 818–824.
17. MITCHELL, A. R., ERICKSON, B. W., RYABTSEV, M. N., HODGES, R. S., AND MERRIFIELD, R. B. (1976) *J. Amer. Chem. Soc.* **98,** 7357–7362.
18. TAM, J. P., HEATH, W. F., AND MERRIFIELD, R. B. (1983) *Int. J. Peptide Protein Res.* **21,** 57–65.
19. KASHYAP, M. L., HYND, B. A., ROBINSON, K., AND GARTSIDE, P. S. (1981) *Metabolism* **30,** 111–118.
20. MATSUOKA, N., SHIRAI, K., AND JACKSON, R. L. (1980) *Biochim. Biophys. Acta* **620,** 308–316.
21. JACKSON, R. L., TAJIMA, S., YAMAMURA, T., YOKOYAMA, S., AND YAMAMOTO, (1986) *Biochim. Biophys. Acta* **875,** 211–219.
22. KASHYAP, M. L., SRIVASTAVA, L. S., CHEN, C. Y., PERISUTTI, G., CAMPBELL, M., LUTMER, R. F., AND GLUECK, C. J. (1977) *J. Clin. Invest.* **60,** 171–180.

23. BARANY, G., AND MERRIFIELD, R. B. (1980) *in* The Peptides (Gross, E., and Meienhofer, J., Eds.), Vol. 2, pp. 1–284, Academic Press, New York.
24. KENT, S. B. H., MITCHELL, A. R., ENGLEHAND, M., AND MERRIFIELD, R. B. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2180–2184.
25. MERRIFIELD, R. B., VIZIOLI, B. D., AND BOMAN, H. G. (1982) *Biochemistry* **21,** 5020–5031.
26. KONIG, W., AND GEIGER, R. (1970) *Chem. Ber.* **103,** 788–798.
27. TAM, J. P., HEATH, W. F., AND MERRIFIELD, R. B. (1983) *J. Amer. Chem. Soc.* **105,** 6442–6455.