Validation of a "clearing" assay for milk lipoprotein lipase in agarose gel

DANA E. WILSON, CAROLYN M. FLOWERS, AND JAMES C. READING

Departments of Medicine and Community and Family Medicine, The University of Utah, College of Medicine, Salt Lake City, Utah 84112

Summary We have developed a simplified method for the quantitative assay of lipoprotein lipase in cow's milk based on the hydrolysis of a glyceride emulsion in semisolid agarose gel. The area of clearing produced thereby is a function of enzyme concentration. Absolute molar rates for unknown samples may be calculated if standards of known activity are used concurrently. The precision of the simplified assay compared favorably with a method based on titrimetric determination of the rate of free fatty acid release. A modified assay has been used to assess the potency of lipoproteins in lipoprotein lipase activation.

Supplementary key words triglyceride · fatty acid · milk · clearing factor

THE LIPOPROTEIN LIPASE of cow's milk has been of considerable physiological and biochemical interest. The mammary gland extracts glycerides from maternal plasma during lactation, a process which appears to involve initial hydrolysis by LPL (1, 2). Maternal plasma glyceride fatty acids furnish a substantial proportion of milk glyceride fatty acids in some species and, thus, LPL may play an important role in neonatal nutrition (1). LPL has been purified from cow's milk by affinity chromatography (3). Cow's milk has been used in a test system for peptide and lipoprotein activators of LPL because it is devoid of apolipoprotein LPL activators (4, 5). In these studies LPL activity was determined by the tedious and time-consuming measurement of the rate of release of glycerol or free fatty acids, the products of triglyceride hydrolysis. Because of its potential usefulness, we report here a greatly simplified quantitative assay system for milk LPL based on the clearing of a glyceride emulsion in a semisolid medium.

The following materials were used in the plate-clearing assay: agarose (Sargent-Welch Scientific Co., Skokie, Ill.); bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.); Intralipid, 20% (Vitrum, Stockholm), containing fractionated soybean oil, 20 g/l; egg lecithin, 12 g/l; glycerol, 25 g/l; and disposable square plastic petri dishes, 100 × 15 mm (Lab-Tek Products, Westmont, Ill.). Citrated plasma was obtained from the University Hospital Blood Bank and was dialyzed against 0.9% sodium chloride, pH 9.0, prior to use. Unpasteurized cow's milk was obtained from the Hi-Land Dairy, Salt Lake City, Utah. In the majority of the assays, milk was skimmed by centrifugation (1.9×10^5 *g*-min at the tube tip, R_{max}), dialyzed against sodium

Abbreviations: LPL, lipoprotein lipase; REU, relative enzyme activity; VLDL, very low density lipoproteins.

citrate, 0.02 M, sodium chloride, 0.1 M, pH 7.0, for 2 hr, and then against 0.005 M barbital, 0.157 M sodium chloride, pH 7.4, for 2 hr with two changes of buffer in each instance. The dialyzed milk was then ultracentrifuged in polycarbonate tubes (2.7×10^6 *g*-min at R_{max}) and the clear middle layer was separated with a suction pipette. Multiple small portions of a single batch of skimmed dialyzed unpasteurized milk of known activity were frozen at −20°C and were thawed for standards in each plate. Very low density lipoproteins were separated from fresh citrated lipemic bank blood at plasma density (1.006 g/ml) by ultracentrifugation (1×10^8 *g*-min at R_{max}). The tubes were then sliced and the top fractions were collected by aspiration. VLDL protein concentration was measured by the method of Lowry et al. (6) against a bovine serum albumin standard after ether extraction (7).

Except where stated otherwise, assay plates were made in the following manner. Stock agarose, 2%, was made in 0.2 M ammonium chloride buffer, pH 8.6, with 0.1% sodium azide. Immediately prior to use, the agarose was melted at 100°C in a water bath. In a separate tube, we introduced: albumin, 10.6% in Tris buffer, 0.005 M, pH 8.6, 10 ml; Tris buffer, 0.005 M, pH 8.6, 8 ml; Intralipid, 20%, 0.06 ml; and dialyzed plasma, 0.3 ml. This mixture was maintained at 50°C in a water bath, and 6.5 ml of melted 2% agarose was added with mixing. The plate was poured and allowed to gel and to stand for 18–24 hr prior to use. Holes were punched in the agar gel with a specially constructed punch (Design and Fabricating Co., Salt Lake City) producing a well with a 4-mm diameter. A 20-μl sample for enzyme assay was pipetted into each well. The plates were then allowed to incubate in a humidity chamber at room temperature (24°C). After 72–96 hr, when equilibrium clearing was approached (Fig. 1), the plates were photographed on Polaroid direct positive film, and the diameters of the completely cleared zones were measured in several dimensions to the nearest 0.1 mm by a single observer. A centimeter ruler was photographed with each plate for the calculation of photographic magnification.

The area (A) of the cleared zone (mm^2) was calculated from the equation:

$$A = \frac{\pi}{M}\left[\left(\frac{\Sigma_i D_i}{2N}\right)^2 - \left(\frac{\Sigma_j d_j}{2n}\right)^2\right]$$

where d_j is the jth measurement of the diameter of the sample well, D_i is the ith measurement of the diameter of the clear zone, M is the photographic magnification, and N and n are the numbers of sample well and clear zone diameter measurements, respectively. Computations were performed on the Olivetti Programma 101 desk computer.

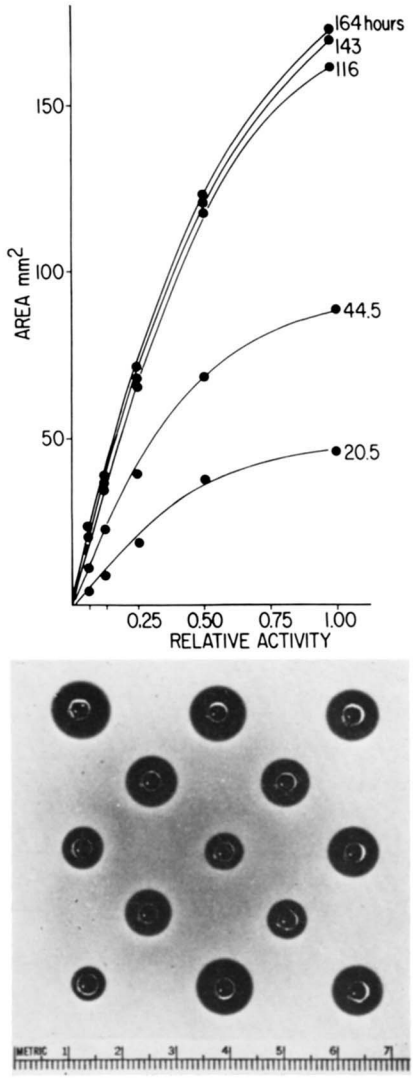

FIG. 1. Typical standard curves for plate-clearing assay of dilutions of milk LPL. Relative enzyme activity of the stock standard was taken as 1.00. Elapsed incubation time is noted by each curve.

Relative enzymatic activity by plate-clearing was determined from a standard curve prepared for each plate by dilutions of 1/2, 1/4, 1/8, and 1/16 of the stock frozen standard in barbital buffer (Fig. 1). Relative enzyme activity for unknowns was determined either visually or by computer from this curve. Curve fitting was performed on the UNIVAC 1108 computer (Biomedical Program BMDO6R) at the University of Utah. The best fit with experimental data (standard curves with known dilutions of enzyme) was found with an exponential equation of the form: $y = A + BR^x$, where y = relative enzyme activity and x = area of clearing. The sum of the squares of the residuals was about 0.1 REU^2. Polynomial equations of second, third, and fourth degree did not fit the data as well. Relative activity of the undiluted stock standard was taken as 1.00. The absolute

activity of the unknown was then calculated as: relative activity of unknown × absolute activity of standard (μmoles ml^{-1} min^{-1}) = absolute activity of unknown (μmoles ml^{-1} min^{-1}).

Incubation–fatty acid titration assays were performed essentially as described by Boberg (8) for comparative purposes and to establish the absolute activity for the milk LPL standard used on each plate.

Assay conditions. An alkaline pH optimum for milk LPL has been found by ourselves and other groups in standard assays. This was assumed to apply to this system and was not investigated further. No clearing occurred at 5°C. At 37°C there was rapid initial clearing but ultimate zone size was less than at 24°C. Therefore, 24°C, the ambient laboratory temperature, was chosen for the remaining studies.

No clearing was observed in the absence of serum activator or lipoproteins or in the absence of NH_4^+ when plates were made in Tris buffer, 0.005 M, pH 8.6. Plates made 1.0 M in sodium chloride showed no clearing. Albumin, agarose, Intralipid, and plasma concentrations were selected to give large and distinct cleared zones (Figs. 1 and 2). Faint halos often surrounded the distinct cleared zones. Zone size increased with decreasing agarose and Intralipid concentrations (Fig. 2, *A* and *C*). Agarose concentrations of less than 0.5% resulted in plates with insufficiently rigid wells, and the definition of cleared zones from the remainder of the plate became poor at very low Intralipid concentrations. Increasing zone size was observed with increasing albumin concentration. Albumin at concentrations greater than 5% inhibited clearing, but systematic examination of other albumin sources to eliminate this effect was not undertaken (Fig. 2*B*). Under usual assay conditions as described above, cleared zones appeared within 6 hr, enlarged rapidly for the next 48 hr, and reached equilibrium between 72 and 96 hr (Fig. 1). Similar results were obtained with whole unpasteurized milk, skimmed milk, and milk dialyzed against sodium citrate to disrupt casein micelles (3).

Reliability of assay. Within a single plate, analysis of replicate samples of milk LPL showed a coefficient of variation of 0.036, n = 13, a degree of precision which compares favorably with the analytical error for glycerol and free fatty acid determinations (8). For purposes of comparison with a traditional assay method, serially diluted samples of milk were tested for LPL activity by both incubation–titration and plate-clearing methods (n = 50). The correlation between methods was good (Fig. 3). The correlations between calculated dilution and measured relative activity for the plate-clearing and incubation–titration assays were 0.968 and 0.946, respectively. Normalized predicted activities for diluted samples (measured activity × dilution^{-1} × activity of undiluted standard^{-1}) were 1.07 ± 0.32 SD and 1.03 ± 0.15 SD for the incubation–titration and plate-clearing assays, respectively. The coefficient of variation for the normalized data in the incubation–titration assay (0.30) was twice that for the plate-clearing assay (0.15). These results indicate that the plate-clearing assay is at least as

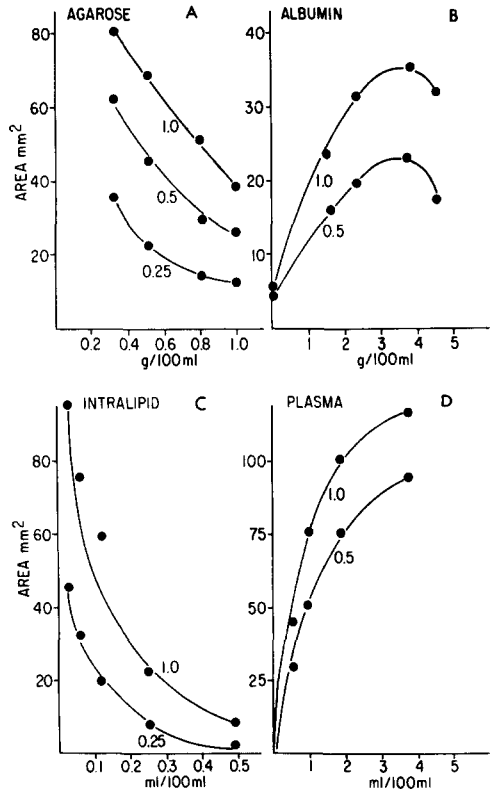

FIG. 2. Effect of varying individual components in the standard assay system. Separate curves refer to dilutions of stock milk LPL. Concentrations are expressed as weight or volume/100 ml.

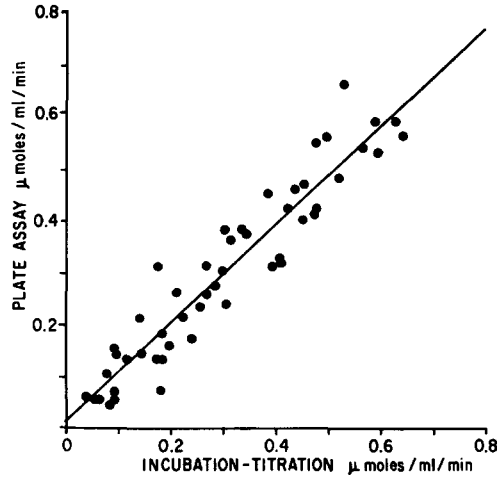

FIG. 3. Correlation between LPL activity by incubation–fatty acid release and plate-clearing assays for samples of milk diluted in NH_4Cl buffer, pH 8.6. n = 50, r = 0.938.

precise as a method based on measurement of free fatty acid release. A direct comparison between the results of the plate-clearing assay and glycerol release, a method offering improved precision over fatty acid titration in the assay of human plasma postheparin lipolytic activity (8), was not made.

A modification of this assay method has permitted measurement of the potency of lipoprotein fractions in the activation of LPL (Fig. 4). A standard assay mixture was prepared without plasma, and milk LPL was added at 50°C with rapid cooling to minimize thermal denaturation of the enzyme. Bovine milk LPL activity is negligible in the absence of plasma or lipoprotein activators (4). As expected, without VLDL to activate LPL, no clearing was observed. Dilutions of VLDL in buffer were pipetted into the wells, and the plate was allowed to incubate as in the standard assay. The resultant cleared zones were less distinct than in the standard assay but were measurable. The apparent K_m for activation by VLDL was 7.3 μg/ml, a value similar to our own results based on fatty acid release and to published values (4).

In early studies, workers attempted to measure LPL activity by determining the rate of optical clearing of glyceride emulsions activated by plasma or lipoproteins (9). Although these assays were rapid and convenient, they were abandoned for several reasons. First, activity was not expressed in absolute molar terms, but rather in arbitrary absorbance units. Secondly, since triglyceride hydrolysis has been thought to proceed through cleavage of each of three glyceride ester bonds and to involve the sequential formation of di- and monoglycerides, optical clearing has been thought to be a poor index of enzymatic activity on theoretical grounds. The introduction of chemical, radioisotopic, and enzymatic methods for the determination of rates of free fatty acid and glycerol release has largely eliminated these objections but has greatly increased the technical effort and time involved in LPL assay.

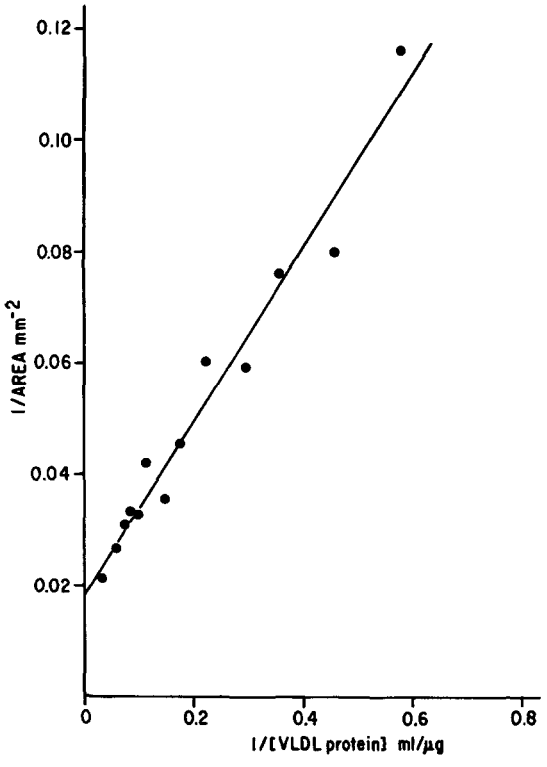

FIG. 4. Lineweaver-Burk plot of the apparent K_m for activation of the milk LPL system by human VLDL. The standard assay gel contained milk LPL (5 ml) but no plasma. The wells contained dilutions of VLDL. The correlation coefficient for the double reciprocal plot was 0.982, n = 13.

Scanu and Page (10) reported the detection of LPL in postheparin plasma by clearing of a coconut oil emulsion in agar. Our data show that quantitation of an assay based on this principle is feasible. Objections to the use of a "clearing" assay for bovine milk LPL appear to have been nullified by the present technique. Relative enzymatic activity can be calculated from the area of the cleared zone and a curve of known dilutions of standard. If the absolute activity, determined by an independent method, is known for one or more of the standards, then absolute activity (μmoles ml^{-1} min^{-1}) can be estimated for unknown samples. The good correlation between the results of incubation–titration assays and plate-clearing assays suggests that the generation of partial glycerides does not have any major influence on the results of the plate-clearing assay. The precision of the plate-clearing assay compares favorably with the more tedious method for FFA release after incubation. Replicate analyses in a single plate gave a coefficient of variation of 0.036, a result similar to the analytical error for glycerol release (7).

The relationship between zone area and enzymatic activity has been nonlinear and may involve a number of processes including diffusion and enzyme inactivation. The albumin requirement for optimal clearing suggests that there may be end-product inhibition in the absence of a fatty acid acceptor (8). We have not identified the lipid constituents of the faint halos which often surround the completely cleared zones, but it seems likely that these halos contain partial glycerides due to incomplete hydrolysis. For the assay of milk LPL, these data may be treated empirically. A computer fit has simplified the calculation of relative activities, although the latter may be determined satisfactorily by visual inspection of standard curves. Since critical physicochemical properties of LPL such as molecular size, configuration, and diffusibility may differ in preparations from sources other than milk, this technique may not be universally applicable. The plate-clearing assay has greatly simplified the measurement of LPL in bovine milk, however, and seems to be a useful method for the assay of large numbers of

stable fractions during enzyme purification or for the determination of the LPL-activating properties of lipoprotein apoglycopeptides.

The authors wish to thank Jane Newby, Robert James, and Gregory Done for skilled technical assistance, and Mr. Delbert Egan for providing us with milk samples.

This work was supported in part by U.S. Public Health Service grants HE 13626 and AM-00002. Dr. Wilson is an investigator of Howard Hughes Medical Institute.

Manuscript received 18 May 1972; accepted 11 September 1972.

REFERENCES

1. Linzell, J. L., and M. Peaker. 1971. Mechanism of milk secretion. *Physiol. Rev.* **51:** 564–597.
2. Hamosh, M., T. R. Clary, S. S. Chernick, and R. O. Scow. 1970. Lipoprotein lipase activity of adipose and mammary tissue and plasma triglyceride in pregnant and lactating rats. *Biochim. Biophys. Acta.* **210:** 473–482.
3. Olivecrona, T., T. Egelrud, P.-H. Iverius, and U. Lindahl. 1971. Evidence for an ionic binding of lipoprotein lipase to heparin. *Biochem. Biophys. Res. Commun.* **43:** 524–529.
4. Havel, R. J., V. G. Shore, B. Shore, and D. M. Bier. 1970. Role of specific glycopeptides of human serum lipoproteins in the activation of lipoprotein lipase. *Circ. Res.* **27:** 595–600.
5. Korn, E. D. 1962. The lipoprotein lipase of cow's milk. *J. Lipid Res.* **3:** 246–250.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
7. Brown, W. V., R. I. Levy, and D. S. Fredrickson. 1969. Studies of the proteins in human plasma very low density lipoproteins. *J. Biol. Chem.* **244:** 5687–5694.
8. Boberg, J. 1970. Quantitative determination of heparin released lipoprotein lipase activity in human plasma. *Lipids.* **5:** 452–456.
9. Korn, E. D. 1959. The assay of lipoprotein lipase *in vivo* and *in vitro*. *Methods Biochem. Anal.* **7:** 145–192.
10. Scanu, A., and I. H. Page. 1959. Separation and characterization of human serum chylomicrons. *J. Exp. Med.* **109:** 239–256.