Development of a Membrane-Based Assay for Detection of Arginase: Application to Detection of Arginine-Binding Proteins in Peanuts

Si-Yin Chung* and Yvette M. Bordelon

Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1100 Robert E. Lee Boulevard, P.O. Box 19687, New Orleans, Louisiana 70179

A procedure employing a conjugate of arginine–6-aminohexanoate–biotin–avidin–horseradish peroxidase (Arg–LC–biotin–avidin–HRP) to detect arginase or arginine-binding proteins on a poly(vinylidene difluoride) (PVDF) membrane is described. For development of the method, a commercial arginase was used as the model system. Steps for detection include subjecting the arginase to gel electrophoresis and electrotransferring to a PVDF membrane where the arginase was detected with Arg–LC–biotin–avidin–HRP and visualized as a colored band after incubation with a HRP substrate. Requirements for successful detection include use of a spacer arm (i.e., –LC–) inserted in an Arg–HRP conjugate and a buffer (sodium borate) which does not interact with manganese chloride ($MnCl_2$) to form precipitates during incubation. $MnCl_2$ is required for activating and facilitating the arginase to bind to Arg–LC–biotin–avidin–HRP. The conjugate was prepared through a sequential reaction between arginine, *N*-(hydroxysuccinimido)-6-aminohexanoate–biotin (NHS–LC–biotin), and avidin–HRP. The sensitivity of the assay was 2 μg of arginase on the membrane. The Arg–LC–biotin–avidin–HRP also detected ovalbumin, arginine decarboxylase, and kinase except for bovine serum albumin and alkaline phosphatase. Application of the method to a crude peanut extract revealed several proteins having an affinity for arginine. The role of these arginine-binding proteins in reduction of the free arginine level in peanuts is discussed.

INTRODUCTION

The relationship between arginine and peanut maturity has been reported (Young et al., 1973; Basha et al., 1976). Usually, free arginine decreases significantly in level with peanut maturity. Because of this phenomenon, arginine has been used as the maturity index for prediction of peanut harvest dates and maturity (Young and Mason, 1972; Fincher et al., 1980). To date, little information is available regarding the cause of the decrease of free arginine in mature peanuts. It is postulated that the decrease could be due to an increased activity of arginase, a metal ion-activated enzyme (Kuhn et al., 1991; Patchett et al., 1991) which converts L-arginine to L-ornithine and urea. Another possibility is that arginine could be bound to peanut proteins (i.e., arginine-binding proteins), therefore restricting it from being free. This ability of arginine to bind to proteins/enzymes has been demonstrated in cases where arginine was used as a ligand for purification of arginase (Kang and Cho, 1990), aminopeptidase (Khald and Marth, 1990), and fibronectin (Vuento, 1979). Moreover, arginine has been shown to be capable of binding to ribonucleic acid (RNA) or nucleotides (Puglisi et al., 1992).

To verify the above assumption that arginase or arginine-binding proteins are present in peanuts, an analytical technique such as the membrane-based assay which can detect both enzyme and proteins at the same time is needed. The assay is similar to a Western blot procedure (Towbin et al., 1979; Simonin et al., 1991; Buee et al., 1991) except that an arginine–peroxidase conjugate was used instead of an antibody–peroxidase conjugate to detect the target proteins and enzyme. Because arginase from soybean has been shown to be capable of binding to arginine immobilized onto agarose gel (Kang and Cho, 1990), a commercial L-arginase (EC 3.5.3.1) was used as the model system. In the method, arginase was subjected to gel electrophoresis, followed by electrotransferring to a PVDF membrane, where it was detected by an arginine–peroxidase conjugate and visualized as a colored band after incubation with a peroxidase substrate. In this study, we report on the conditions for successful detection of arginase on PVDF membrane and the applicability of the method to detecting arginine-binding proteins/arginase in peanuts.

MATERIALS AND METHODS

Materials. Precast 4–20% Tris–glycine gradient gels without sodium dodecyl sulfate (SDS) and nitrocellulose membranes (BAS-83 and PH79) were purchased from Schleicher & Schuell (Keene, NH). PVDF membranes and Immobilon P were purchased from Bio-Rad Laboratories (Richmond, CA) and Millipore Corp. (Bedford, MA). Alkaline phosphatase (AP) was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). L-Arginine (free base), L-arginase, bovine serum albumin (BSA), extravidin–alkaline phosphatase, avidin–peroxidase, glutaraldehyde (GL), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT), and 4-chloro-1-naphthol were supplied by Sigma Chemical Co. (St. Louis, MO). Activated horseradish peroxidase (HRP) and *N*-(hydroxysuccinimido)-6-aminohexanoate–biotin (NHS–LC–biotin) were obtained from Pierce (Rockford, IL). L-(+)-Canavanine sulfate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Enzygraphic Web was purchased from VWR Scientific (New Orleans, LA). BLAST blot amplification systems were obtained from Du Pont NEN Research Products (Boston, MA). Mature and immature physiologically selected Florunner peanuts (*Arachis hypogaea* L.) were obtained from the U.S. Department of Agriculture (USDA) National Peanut Research Laboratory (Dawson, GA).

Preparation of Arginine–Enzyme Conjugates. *Arginine–Horseradish Peroxidase (Arg–HRP).* The conjugate was prepared by incubating at 4 °C a mixture of 100 μL of arginine (34 mg/mL) in 1 M bicarbonate, pH 9.5, and 100 μL of activated HRP (20 mg/mL) in deionized-distilled water for 16 h, followed by quenching with 60 μL of 0.2 M glycine for 2 h at 25 °C and adding 700 μL of a 1.4% BSA solution. The resultant conjugate was then dialyzed against 0.01 M Tris, pH 7.5, and stored at 4 °C with thimerosal (a preservative to keep bacteria from growing) added at a final concentration of 0.1%.

Arginine-6-Aminohexanoate–Biotin–Avidin–Horseradish Peroxidase (Arg–LC–Biotin–Avidin–HRP). The conjugate was prepared by covalently attaching arginine to NHS–LC–biotin,

This article not subject to U.S. Copyright. Published 1993 by the American Chemical Society

followed by addition of avidin-HRP. Briefly, NHS-LC-biotin (1 mg) was added to a 1-mL solution of excess arginine (10 mg/mL) in 0.05 M bicarbonate, pH 8.5. The mixture was incubated in ice for 2 h, followed by addition of avidin-HRP (2 mg) and continued incubation for 1 h at 4 °C. BSA was added at a final concentration of 1%. The resultant conjugate was then dialyzed against 0.01 M Tris, pH 7.5, and stored at 4 °C with thimerosal added.

Arginine-6-Aminohexanoate-Biotin-Avidin-Alkaline Phosphatase (Arg-LC-Biotin-Avidin-AP). The conjugate was prepared in the same way as the Arg-LC-biotin-avidin-HRP except that 0.5 mL of extravidin-alkaline phosphatase (1.5 mg/mL) was added.

Arginine-Glutaraldehyde-Alkaline Phosphatase (Arg-GL-AP). The conjugate was prepared by incubating a mixture of 10 μL of 25% glutaraldehyde (GL) and a 1.5-mL solution of alkaline phosphatase (2 mg/mL) in 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ for 1.5 h at 25 °C, followed by addition of 20 mg of arginine and incubation for another 1.5 h. BSA was added, and the resultant conjugate was dialyzed against 0.01 M Tris, pH 7.5, and stored at 4 °C with thimerosal added.

Canavanine-6-Aminohexanoate-Biotin-Avidin-Horseradish Peroxidase (Can-LC-Biotin-Avidin-HRP). The conjugate was prepared in the same way as the Arg-LC-biotin-avidin-HRP except that 15 mg of canavanine (an analog of arginine) was used instead of arginine.

Gel Electrophoresis and Electrotransfer of Arginase. Both polyacrylamide gel electrophoresis and electrotransfer were performed according to the instructions of the manufacturer (Schleicher & Schuell) using a Profile mini-electrophoresis system. Electrotransfer is a process in which the transfer of proteins from gels to membranes is achieved by applying an electric field to the gel-membrane sandwich which is placed between absorbent paper soaked in transfer buffer. L-Arginase (120 000) (10 μL per well; 20 μg each) was run on a precast 4-20% Tris-glycine gradient non-SDS gel (10 × 1.0 mm), followed by electrotransfer to a PVDF membrane (Immobilon P or Bio-Rad). For sensitivity determination, arginase at various amounts (1, 2, 4, and 8 μg; 10 μL each) was applied. Gels and membranes were stained with Coomassie Brilliant Blue R-250 and Amido Black, respectively.

Detection of Arginase. After electrotransfer, membrane was blocked with 3% bovine serum albumin (BSA) in 50 mM Tris buffer, pH 8.0, for 2 h at 25 °C, followed by three washes (10 min each) with 10 mM sodium borate-Tween 20 (0.1%), pH 9.0. The purpose of blocking is to prevent nonspecific binding of arginine-enzyme conjugate to the membrane by saturating the unoccupied sites on the membrane with protein such as BSA. The membrane was then incubated in borate-$MnCl_2$-Tween 20, pH 9.0, for an hour with the arginine-enzyme conjugate at a dilution of 1:500, followed by three washes with borate-Tween 20. The arginase on the membrane was visualized as a colored band after incubation for 15 min with a substrate solution [e.g., 4-chloro-1-naphthol for horseradish peroxidase or 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) for alkaline phosphatase; for preparation of substrate, see manufacturer's manual].

Preparation and Assay of Crude Protein/Arginase Extract from Peanuts. Mature or immature physiologically selected peanuts were shelled, deskinned, and ground in cold acetone. The resultant acetone powders were air-dried. Crude protein extract was prepared by allowing the acetone powders (20 g) to stir overnight at 4 °C in 0.01 M sodium borate (200 mL), pH 7.4, containing 0.2 M NaCl, 1 mM $MnCl_2$, and 10% glycerol. The mixture was centrifuged and the supernatant subjected to 40 and 85% ammonium sulfate fractionations. The 85% fraction was collected, dialyzed, concentrated, and analyzed for arginine-binding proteins and arginase using the developed membrane-based assay. Soluble arginase activity was determined by measurement of urea according to the method of Oginsky (1957). Protein concentrations were determined using the Bio-Rad protein assay.

RESULTS AND DISCUSSION

Optimal Conditions for Detection of Arginase. *Selection of Borate Buffer Containing $MnCl_2$.* The prerequisite for binding of Arg-LC-biotin-avidin-HRP to arginase blotted onto a PVDF membrane is to have both incubated for a period of time in a desired buffer containing $MnCl_2$, which is required for activation of arginase. Therefore, the buffer to be selected should have no interaction with $MnCl_2$, which is known to precipitate in phosphate buffer during incubation (Kang and Cho, 1990; Johnsen, 1991). Precipitation of $MnCl_2$ results in the failure to detect arginase on the membrane (see below). In view of this, several buffers such as Tris, glycine, Hepes, carbonate, diethanolamine, phosphate, and borate were respectively tested for their reactivity toward $MnCl_2$. All but borate were found to induce $MnCl_2$ precipitation within 1 h of incubation.

Investigation of the effect of $MnCl_2$ precipitation on the assay showed that precipitation of $MnCl_2$ resulted in a negative assay. Here, a negative assay is defined as one in which a band representing arginase does not appear on the membrane (for details of detection, see Materials and Methods). Results showed that only borate gave a positive response in the assay, while no bands were detected with buffers forming precipitates with $MnCl_2$. The absence of bands with the latter suggested that the arginase on the membrane was unable to bind to Arg-LC-biotin-avidin-HRP due to lack of activation by $MnCl_2$.

Borate was, therefore, selected as the bufffer for $MnCl_2$ because it did not form precipitates with $MnCl_2$ during incubation and did give a positive assay.

Insertion of a Spacer Arm in Arg-HRP Conjugate. During the course of the study, it was noted that incubation of the membrane-bound arginase with Arg-HRP did not give a band on the membrane, regardless of $MnCl_2$ present or absent in the borate buffer. This suggested that binding between the arginase and Arg-HRP probably did not occur and that the failure in binding could be due to some steric hindrance which prevented the arginine moiety of Arg-HRP from binding to the arginase on the membrane.

To verify the above postulation of potential steric hindrance, a spacer arm of 6-aminohexanoic acid (LC), which is known to reduce steric hindrance associated with the binding of biotinylated peptides to avidin (Jans et al., 1990; Smith et al., 1991), was introduced into the Arg-HRP. This was done by reacting arginine with NHS-LC-biotin to form Arg-LC-biotin, followed by attaching the latter to an avidin-HRP conjugate. The resultant conjugate was an Arg-LC-biotin-avidin-HRP. When this was added to the membrane-bound arginase activated by $MnCl_2$ in borate buffer, binding between the conjugate and arginase occurred. This was confirmed by the appearance of a band on the membrane following the addition of a HRP substrate. The result suggests that a spacer arm inserted in the Arg-HRP is indeed essential for removing steric hindrance and facilitating the binding between arginase and the arginine moiety of the conjugate.

Use of BSA and Tween 20 in Separate Steps To Lower Membrane Background. In an attempt to lower the background of arginase-bound membranes, different agents [BSA, non-fat dry (NFD) milk, and Tween 20] were incorporated separately into various incubation steps: (1) agent A, for incubation with arginase-bound membrane before the addition of Arg-LC-biotin-avidin-HRP; (2) agent B, for incubation with agent A-blocked membrane as well as borate-$MnCl_2$ containing Arg-LC-biotin-avidin-HRP. The combined effects of agents A and B (expressed as A/B) in those two steps on the background and assay were then evaluated.

Of the combinations shown in Table I, BSA/Tween 20 was the most effective in lowering the background and

Table I. Effect of Blocking Agents on Membrane Background and Detection

agent A[a]/agent B[b]	membrane background	detection[c]
BSA/BSA	high	–
BSA/NFD milk	no	–
BSA/Tween 20	low	+
NFD milk/Tween 20	no	–
Tween 20/Tween 20	high	+

[a] Used for blocking arginase-bound membrane prior to incubation with Arg–LC–biotin–avidin–HRP. Concentrations of BSA, non-fat dry (NFD) milk, and Tween 20 were 3, 3, and 0.05%, respectively. [b] Incubated with Arg–LC–biotin–avidin–HRP and agent A-blocked membrane. [c] + indicates band on membrane; – indicates no band.

gave a positive assay. Other combinations such as BSA/NFD milk and NFD milk/Tween 20 failed to give positive results, suggesting that NFD milk interfered with the assay. Although NFD milk may inhibit the interaction of biotin and avidin (Hoffman and Jump, 1989), it seems unlikely that the failure of this assay was due to the effect of NFD milk on the binding between Arg–LC–biotin and avidin–HRP. This is because the biotin–avidin complex (i.e., Arg–LC–biotin–avidin–HRP) had been formed before NFD milk was added, and once it was formed, the complex should hardly be disturbed by extremes of pH, salts, and chaotropic agents (Wilcheck and Bayer, 1988).

Another observation was that high background was seen with the combinations of BSA/BSA and Tween 20/Tween 20 (Table I). However, only the former gave a negative assay, indicating that BSA interfered with the assay. Because $MnCl_2$ is known to bind to BSA (Peters, 1985), the interference with the assay by BSA was thought to be a result of BSA–$MnCl_2$ interaction, which led to $MnCl_2$ being inaccessible to the arginase on the membrane and subsequently the arginase not activated and unable to bind to Arg–LC–biotin–avidin–HRP. The BSA–$MnCl_2$ interaction appeared to occur only when BSA (i.e., agent B) was free. If BSA (agent A) was membrane-bound and agent B was Tween 20 instead of BSA, the assay became positive (Table I), indicating that bound BSA had no effect on $MnCl_2$ and, as a result, $MnCl_2$ remained free in the solution and capable of activating and facilitating the arginase to bind to Arg–LC–biotin–avidin–HRP. Since BSA/Tween 20 gave the best performance among all combinations, it was used for the rest of the studies.

Use of Arg–LC–Biotin–Avidin–HRP on Immobilon P Membrane. In addition to Arg–LC–biotin–avidin–HRP, three additional enzyme conjugates, Can–LC–biotin–avidin–HRP, Arg–LC–biotin–avidin–AP, and Arg–GL–AP, were prepared. Their effects on membrane background and band intensity were compared (Figure 1). Canavanine (Can) was used because it is an analog of arginine and also a substrate for arginase (Rosenthal, 1990). Alkaline phosphatase (AP) was employed because the color of the band it produces does not fade when exposed to light, whereas in the case of HRP the color fades with time. Glutaraldehyde (GL) was used because it provides a spacer arm, the effect of which on membrane background was compared with that of the –LC– spacer.

Results on the conjugates' performance on a PVDF membrane such as Immobilon P (Figure 1, top lanes) indicated that both Arg– and Can–LC–biotin–avidin–HRP performed equally well and were lower in background than Arg–LC–biotin–avidin–AP and Arg–GL–AP. The HRP conjugates were superior in this case due to the fact that they produced a background that faded easily while the band intensity was retained. By contrast, the background with both AP conjugates did not fade, thus affecting the visibility of the band on the membrane.

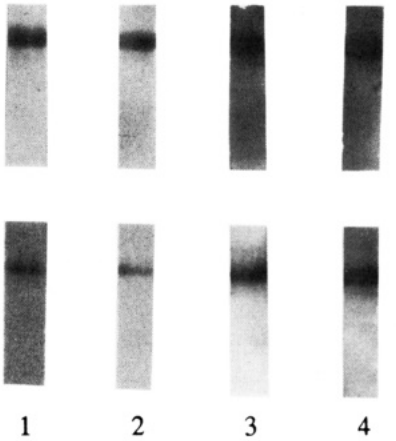

Figure 1. Detection of arginase (20 μg) on PVDF membranes with various enzyme conjugates: (top lanes) Immobilon P membrane; (bottom lanes) Bio-Rad PVDF membrane; (lane 1) Can–LC–biotin–avidin–HRP; (lane 2) Arg–LC–biotin–avidin–HRP; (lane 3) Arg–LC–biotin–avidin–AP; (lane 4) Arg–GL–AP.

Despite having a background problem with Immobilon P, the AP conjugates appeared to perform well with another commercial membrane, the Bio-Rad PVDF membrane. As shown in Figure 1 (bottom lanes), background on this membrane was much lower compared to that on Immobilon P (Figure 1, top lanes). In this case, AP conjugates were superior because they gave thicker and darker bands than HRP conjugates on the Bio-Rad membrane. On the other hand, Arg–LC–biotin–avidin–AP was preferred over Arg–GL–AP due to the fact that the former gave a lower background. This difference in background between the AP conjugates could be due to the type of spacer arm (i.e., –LC– or –GL–) each conjugate carried.

In a further effort to determine the type of membrane which works best for the conjugates, two nitrocellulose membranes, PH79 and BAS-83, differing in pore size were tested. It was found (data not shown) that despite good background, both BAS-83 and PH79 membranes were incapable of giving a band (thick and dark) comparable to that on Immobilon P or Bio-Rad PVDF membrane, regardless of the type of the enzyme conjugate used (i.e., HRP or AP conjugate). This inferiority of nitrocellulose to PVDF membrane has also been demonstrated by Domingo and Marco (1989) and Buee et al. (1991) and may arise from the use of Tween 20, which is said to disrupt protein binding to the nitrocellulose (Smith et al., 1989).

On the basis of the above observation, it is concluded that PVDF membranes are preferred over nitrocellulose membranes and that to achieve good background and a band of desired intensity, HRP conjugates should be used on Immobilon P membrane and AP conjugates on Bio-Rad PVDF membrane.

Sensitivity and Reactivity with Other Proteins. The sensitivity of the assay was determined by applying various amounts of arginase (1, 2, 4, and 8 μg) to the gel, followed by electrotransferring of the enzyme to Immobilon P membrane (or Bio-Rad PVDF membrane) and detection with Arg–LC–biotin–avidin–HRP (or Arg–LC–biotin–avidin–AP). In each case, the lowest amount of arginase the conjugate could detect was 2 μg (Figure 2). This sensitivity is comparable to that for the detection of lipase and cholesterol esterase from microbes using antibody conjugates (Brahimi-Horn et al., 1991).

In an effort to increase the sensitivity, an Enzygraphic Web detection system was employed. According to the manufacturer (International Biotechnologies, Inc.), the

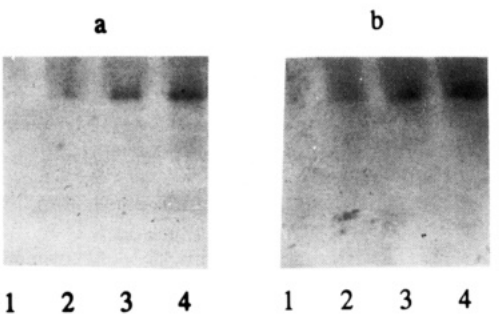

Figure 2. Sensitivity of membrane-based assay: (a) Immobilon P, detected with Arg–LC–biotin–avidin–HRP; (b) Bio-Rad PVDF membrane, detected with Arg–LC–biotin–avidin–AP. Lanes 1, 2, 3, and 4 are 1, 2, 4, and 8 μg of arginase, respectively.

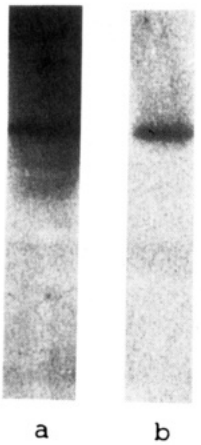

Figure 3. Comparison of assays using (a) the BLAST blot amplification system vs (b) the system developed in this study.

web is a support polymer used to detect HRP-linked probes and reported to be more sensitive than radioactive and existing colorimetric detection methods. In this study, the arginase was detected by placing the web in contact with the PVDF membrane containing the immobilized Arg–LC–biotin–avidin–HRP, and within seconds a band was formed on the surface of the web. Despite its higher sensitivity (1 μg of arinase), the web had problems with extremely high background and rapid fading of the band, which precluded its being photographed.

Additionally, a BLAST blot amplification system (Bobrow et al., 1989, 1991) was also tried to enhance the assay sensitivity. In this BLAST technique, hydrogen peroxide and biotinyltyramide was added to the PVDF membrane containing the immobilized Arg–LC–biotin–avidin–HRP which catalyzed the activation of biotinyltyramide, resulting in the deposition of biotin to the membrane surface. This presumably would increase the signal or band density when a strepavidin–HRP and substrate solution were sequentially added. However, no improvement of the sensitivity was seen using the BLAST system. By contrast, the BLAST system gave a higher background than the present method (Figure 3).

The ability of Arg–LC–biotin–avidin–HRP to bind to other enzymes or proteins was also examined. It was found that, in addition to arginase, the conjugate also bound to arginine decarboxylase and kinase. It did not bind to BSA or alkaline phosphatase (both are non-glycoproteins) but did bind to ovalbumin (a glycoprotein), indicating that the binding to ovalbumin could be due to an ionic interaction between the guanidine group of the arginine moiety of the conjugate and the hydroxyl group of the carbohydrate moiety of ovalbumin. This implies that the present method may also have an application in glycoprotein detection.

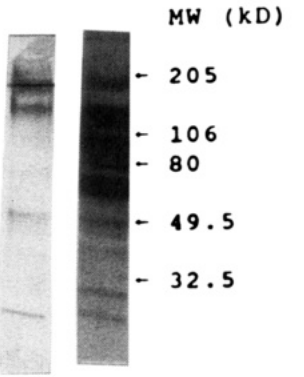

Figure 4. Detection of arginine-binding proteins in peanuts on PVDF membrane: (left lane) proteins detected on Immobilon P membrane with Arg–LC–biotin–avidin–HRP; (right lane) proteins stained on gel with Coomassie Brilliant Blue R-250.

Detection of Arginine-Binding Proteins/Arginase in Peanuts. In an attempt to determine if peanuts contain arginine-binding proteins or arginase, the present membrane-based method was applied to a crude peanut protein extract. Figure 4 (left lane) showed that there were at least four major bands or proteins (approximately 200 000, 150 000, 49 500, and 25 000) strongly binding to Arg–LC–biotin–avidin–HRP and several other proteins appearing as very faint bands. Although no difference in band patterns was found between immature and mature peanuts, protein levels were much higher in the latter (data not shown), suggesting that mature peanuts have more arginine-binding proteins available to bind free arginine, thus resulting in a greater loss of free arginine than immature peanuts.

Additionally, bands on the top of membrane could be arginase (Figure 4, left lane) on the basis of the fact that they have a molecular weight similar to that of arginase (ranging from 110 000 to 240 000) from various sources such as bacteria (Patchett et al., 1991), yeast (Green et al., 1990), and soybean (Kang and Cho, 1990). Besides, activity of soluble arginase was detected (data not shown) in a homogeneous assay which colorimetrically measures urea formed from arginine. Because higher arginase activity was detected in mature peanuts, it is believed that the increased activity could also be the cause of the decrease of free arginine in mature peanuts.

Summary. An assay for detection of arginase or arginine-binding proteins on a PVDF membrane using an Arg–LC–biotin–avidin–HRP conjugate was developed. To achieve successful detection, the following are recommended: (1) use borate buffer as the conjugate diluent and $MnCl_2$ for activation of arginase on the membrane; (2) use BSA for blocking membrane prior to incubation with Arg–LC–biotin–avidin–HRP; (3) after blocking and washing, incubate Arg–LC–biotin–avidin–HRP with the membrane in buffer containing no BSA but Tween 20 and $MnCl_2$; and (4) use Arg–LC–biotin–avidin–HRP on Immobilon P membrane, or use Arg–LC–biotin–avidin–AP on Bio-Rad PVDF membrane. The sensitivity of the assay is 2 μg of arginase on the membrane. The assay can detect ovalbumin but not BSA, indicating that the method may be useful in screening for glycoproteins in some circumstances. Application of the assay to a peanut protein extract revealed several arginine-binding proteins probably associated with the decrease of free arginine in mature peanuts.

ACKNOWLEDGMENT

We thank T. H. Sanders, J. R. Vercellotti, and K. L. Bett for assisting in the harvesting of peanuts in Dawson, GA.

LITERATURE CITED

Basha, S. M.; Cherry, J. P.; Young, C. T. Changes in free amino acids, carbohydrates, and proteins of maturing seeds from various peanut (*Arachis Hypogaea* L.) cultivars. *Am. Assoc. Cereal Chem.* **1976**, *53*, 586–597.

Bobrow, M. N.; Harris, T. D.; Shaughnessy, K. J.; Litt, G. J. Catalyzed reporter deposition, a novel method of signal amplification. Application to membrane immunoassays. *J. Immunol. Methods* **1989**, *125*, 279–285.

Bobrow, M. N.; Shaughnessy, K. J.; Litt, G. J. Catalyzed reporter deposition, a novel method of signal amplification. II. Application to membrane immunoassays. *J. Immunol. Methods* **1991**, *137*, 103–112.

Brahimi-Horn, M. C.; Guglielmino, M. L.; Gaal, A. M.; Sparrow, G. Nondenaturing protein electrotransfer of the esterase activity of lipolytic preparations. *Anal. Biochem.* **1991**, *196*, 76–79.

Buee, L.; Boyle, N. J.; Zhang, L.; Delacourte, A.; Fillet, H. M. Optimization of an alcian blue dot-blot assay for the detection of glycosaminoglycans and proteoglycans. *Anal. Biochem.* **1991**, *195*, 238–242.

Domingo, A.; Marco, R. Visualization under ultraviolet light enhances 100-fold the sensitivity of peroxidase-stained blots. *Anal. Biochem.* **1989**, *182*, 176–181.

Fincher, P. G.; Young, C. T.; Wynne, J. C.; Perry, A. Adaptability of the arginine maturity index method to Virginia type peanuts in North Carolina. *Peanut Sci.* **1980**, *7*, 83–87.

Green, S. M.; Eisenstein, E.; McPhie, P.; Hensley, P. The purification and characterization of arginase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **1990**, *265*, 1601–1607.

Hoffman, W. L.; Jump, A. A. Inhibition of the streptavidin-biotin interaction by milk. *Anal. Biochem.* **1989**, *181*, 318–320.

Jans, D. A.; Bergmann, L.; Peters, R.; Fahrenholz, F. Biotinyl analogues of vasopressin as biologically active probes for vasopressin receptor expression in cultured cells. *J. Biol. Chem.* **1990**, *265*, 14599–14605.

Johnsen, A. H. Nondestructive amino acid analysis at the picomole level of proline-containing peptides using aminopeptidase M and prolidase: application to peptides containing tyrosine sulfate. *Anal. Biochem.* **1991**, *197*, 182–186.

Kang, J. H.; Cho, Y. D. Purification and properties of arginase from soybean, *Glycine max*, Axes. *Plant Physiol.* **1990**, *93*, 1230–1234.

Khald, N. M.; Marth, E. H. Purification and partial characterization of a prolyl-dipeptidyl aminopeptidase from *Lactobacillus helveticus* CNRZ 32. *Appl. Environ. Microbiol.* **1990**, *56*, 381–388.

Kuhn, N. J.; Talbot, J.; Ward, S. pH-Sensitive control of arginase by Mn(II) ions at submicromolar concentrations. *Arch. Biochem. Biophys.* **1991**, *286*, 217–221.

Oginsky, E. L. Isolation and determination of arginine and citrulline. *Methods Enzymol.* **1957**, *3*, 639–643.

Patchett, M. L.; Daniel, R. M.; Morgan, H. W. Characterization of arginase from extreme thermophile *Bacillus caldovelox*. *Biochim. Biophys. Acta* **1991**, *1077*, 291–298.

Peters, T. Serum albumins. *Adv. Protein Chem.* **1985**, *37*, 161–245.

Puglisi, J. D.; Tan, R.; Calnan, B. J.; Frankel, A. D.; Williamson, J. R. Conformation of the TAR RNA-Arginine Complex by NMR Spectroscopy. *Science* **1992**, *257*, 76–80.

Rosenthal, G. A. Metabolism of L-canavanine and L-canaline in leguminous plants. *Plant Physiol.* **1990**, *94*, 1–3.

Simonin, F.; Briand, J. P.; Muller, S.; Murica, G. D. Detection of poly(ADP ribose) polymerase in crude extracts by activity-blot. *Anal. Biochem.* **1991**, *195*, 226–231.

Smith, C. E.; Musich, P. R.; Johnson, D. A. Sodium dodecyl sulfate enhancement of quantitative immunoenzyme dot-blot assays on nitrocellulose. *Anal. Biochem.* **1989**, *177*, 212–219.

Smith, J. S.; Miller, B. T.; Knock, L. S.; Kurosky, A. Biotinylated peptides/proteins. *Anal. Biochem.* **1991**, *197*, 247–253.

Towbin, H.; Staehelin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4350–4354.

Vuento, M. Purification of fibronectin from human plasma by affinity chromatography under non-denaturing conditions. *Biochem. J.* **1979**, *183*, 331–337.

Wilcheck, M.; Bayer, E. A. The avidin-biotin complex in bioanalytical applications. *Anal. Biochem.* **1988**, *171*, 1–32.

Young, C. T.; Mason, M. E. Free arginine content of peanuts (*Arachis hypogaea* L.) as a measure of seed maturity. *J. Food Sci.* **1972**, *37*, 722–725.

Young, C. T.; Matlock, R. S.; Mason, M. E.; Waller, G. R. Effect of harvest date and maturity upon free amino acid levels in three varieties of peanuts. *J. Am. Oil Chem. Soc.* **1973**, *51*, 269–273.

Received for review September 30, 1992. Revised manuscript received December 15, 1992. Accepted December 28, 1992.