Journal of Cellular Biochemistry 32:193–200 (1986)
Proteases in Biological Control and Biotechnology 75–82

Partial Purification of Microsomal Signal Peptidase From Hen Oviduct

R. Keith Baker, Gian Paolo Bentivoglio, and Mark O. Lively

Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27103

Signal peptidase has been purified approximately 600-fold from hen oviduct microsomes. Treatment of microsomes with ice-cold sodium carbonate at pH 11.5 removes soluble and extrinsic membrane proteins prior to solubilization of signal peptidase with Nonidet P-40. After dialysis to pH 8.2, the solubilized enzyme is chromatographed on diethylaminoethyl cellulose at pH 8.2. More than 90% of contaminating proteins bind to the column while signal peptidase and endogenous phospholipid are eluted in the column void volume. Enzyme activity subsequently binds to carboxymethyl cellulose at pH 5.8 and is eluted by approximately 100 to 200 mM NaCl during a NaCl gradient. Polypeptides present in partially purified hen oviduct signal peptidase have relative molecular masses ranging from 54 kD to less than 11 kD with major bands at 29, 23, 22, 19, 18 and 13 kD. The purified peptidase requires phospholipid for activity and is maximally active in the presence of 2 mg/ml phosphatidylcholine.

Key words: hen oviduct, human preplacental lactogen, phosphlipid reactivation, purification, signal peptidase

Hen oviduct signal peptidase (HOSP) is responsible for limited proteolysis of newly synthesized secretory and membrane proteins during their translocation into the lumen of the endoplasmic reticulum [1,2]. Eukaryotic signal peptidases are integral membrane proteins of the endoplasmic reticulum [3–6] and require detergent for solubilization. A phospholipid environment is necessary for enzymatic activity of the solubilized enzymes [7–9]. The genes for two prokaryotic signal peptidases have been isolated from *E coli*: leader peptidase [10] and, prolipoprotein signal peptidase [11]. They are single-chain molecules with relative molecular masses of 36,000 and 18,000, respectively. The prokaryotic peptidases are also membrane proteins [10,12] but do not appear to require phospholipid for activity when solubilized by detergent.

Specific, active site-directed inhibitors of signal peptidase activity have not been identified, so it is not known to which if any of the known mechanistic classes of proteolytic enzymes these enzymes may belong. Dog pancrease signal peptidase has been purified to apparent homogeneity, and its proteolytic activity is tightly associated

Received June 3, 1986, revised and accepted July 22, 1986.

with a complex of from four to six polypeptides [8]. It is not yet known which polypeptide chain(s) of this complex are responsible for proteolysis. We report here the partial purification of a signal peptidase from the magnum region of hen oviduct.

METHODS

Preparation of Oviduct Microsomes and Solubilized HOSP

Oviduct microsomes were prepared from the magnum regions [13] of laying hens by a simplification of our earlier method [4]. All steps were performed at 4°C unless otherwise indicated. Oviduct tissue (400–500 g) was homogenized for 5 min in 4 L homogenization buffer (0.75 M sucrose, 50 mM triethanolamine·HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol) using a Polytron homogenizer (Brinkmann Instruments). Total oviduct microsomes were present in the postmitochondrial supernatant following centrifugation for 10 min at 10,000g and were pelleted by centrifugation of that supernatant for 60 min at 40,000g.

Extrinsic proteins were removed from the microsomes by treatment with ice-cold sodium carbonate at pH 11.5 [14]. The total microsome pellet was suspended in buffer A (0.25 M sucrose, 50 mM triethanolamine·HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol) to a concentration of 50–60 A_{280} units (as determined by dilution of an aliquot into 5% SDS). Two hundred forty milliliters of the suspension, containing approximately 40 g crude microsomes, were stirred for 30 min in 3.4 L 0.1 M Na_2CO_3, pH 11.5, and the carbonate-treated microsomes were collected by centrifugation for 60 min at 48,000g. The pellets were rinsed with a small volume of buffer A and stored at −90°C.

Solubilized HOSP was prepared from carbonate-treated microsomes that were suspended to a concentration of 30 A_{280} units in buffer A containing 20% (v/v) glycerol. The membranes were solubilized by the addition of 10% (w/v) Nonidet P-40 (NP-40) to a final concentration of 2.5 % (w/v) and homogenized with 6–8 strokes of a Dounce homogenizer at room temperature. Solubilized HOSP was recovered in the supernatant following centrifugation for 60 min at 105,000g at 15°C. The detergent extract can be stored at −90°C for more than 1 year without significant loss of activity.

Chromatography of HOSP on Diethylaminoethyl (DEAE) Cellulose

Solubilized HOSP (30–35 ml) was dialyzed overnight against two 2-L changes of DEAE column buffer (10 mM triethanolamine·HCl, pH 8.2, 10% (v/v) glycerol, 5 mM $MgCl_2$, 1% (w/v) NP-40). The solution was centrifuged for 30 min at 105,000g to remove a precipitate that forms during dialysis. The clarified dialysate was applied to a DEAE-cellulose (Whatman DE-52) column (2.5 × 38 cm) equilibrated in DEAE column buffer and eluted with at least two column volumes of the same buffer at a flow rate of 24 ml/hr. HOSP and endogenous phospholipid coelute in a single, broad peak in the unretained fractions. Protein concentrations in the fractions were estimated by a modification of the Lowry method [15], and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed according to Laemmli [16]. Fractions containing the peak of HOSP activity were pooled yielding approximately 90 ml DEAE pool.

Chromatography on Carboxymethyl (CM) Cellulose

The pool obtained from DEAE chromatography was dialyzed overnight against two 4-L changes of CM column buffer (10 mM sodium acetate, pH 5.8, 10% (v/v) glycerol, 0.5% (w/v) NP-40) and then applied to a CM cellulose (Whatman CM-52) column (2.5 × 10.2 cm) equilibrated in the same buffer. The column was washed with 100 ml CM column buffer containing 0.5 mg/ml egg phosphatidylcholine (PC; Avanti Polar Lipids, Inc., Birmingham, AL). HOSP was eluted during a 200-ml linear gradient from 0 to 400 mM NaCl in CM column buffer containing 0.5 mg/ml phosphatidylcholine. Fractions containing HOSP were pooled yielding approximately 45 ml of CM pool.

Signal Peptidase Assay

A translocation-independent assay similar to that described by Jackson [17] was employed using human preplacental lactogen (preHPL) synthesized in the presence of an optimal concentration (3 μg/ml) of antibody specific for mature placental lactogen (HPL) [9]. This substrate was prepared by cell-free protein synthesis in the presence of [^{35}S]methionine ([^{35}S]Met; > 1,000 Ci/mmol, Amersham) using a rabbit reticulocyte lysate system [18] programmed with human placental poly A+ mRNA [19]. Assay conditions for cleavage of preHPL were optimized for each stage of the purification. All assays contained 5 μl translation product and 20 μl HOSP in buffer. Solubilized HOSP was assayed in buffer A containing 2.5% (w/v) NP-40. The DEAE and CM pools were assayed in DEAE column buffer and CM column buffer, respectively. HOSP in CM pool was reactivated by addition of phosphatidylcholine to a final concentration of 2 mg/ml. The reaction products were separated by SDS-PAGE following immunoprecipitation with anti-HPL antibodies that were pre-absorbed onto heat-inactivated, formalin-fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem, La Jolla, CA). Enzyme activity was quantified by densitometry of the resulting autoradiogram [17]. Processing of preHPL to HPL was linear up to about 20% with respect to the amount of HOSP in the assay and was maximal at about 50%. One unit of HOSP activity is defined as that volume of enzyme solution that converts 20% of preHPL to HPL under optimal conditions.

RESULTS

Hen oviduct signal peptidase has been purified using an improved, large-scale method that permits the preparation of up to 90 g (wet weight) of total oviduct microsomes in less than 6 hr. The purification protocol requires four major steps: treatment of microsomes with ice-cold carbonate at pH 11.5, solubilization of carbonate-treated microsomes with NP-40, anion exchange chromatography on DEAE cellulose, and cation exchange chromatography on CM cellulose. A summary of a typical purification appears in Table I, and the polypeptide content and enzymatic activity observed at each stage are shown in Figures 1A and 1B, respectively.

Microsomes are prepared from the magnum regions of oviducts of laying hens. Because tubular gland cells of oviduct magnum have such an extensive endoplasmic reticulum [20], total microsomes prepared according to this protocol contain more than 95% rough microsomes when examined by electron microscopy (data not shown). Therefore, it is not necessary to isolate rough microsomes by centrifugation in sucrose gradients, and the total microsome fraction can be used as the starting material for this procedure.

HOSP is an integral membrane protein, and the protocol takes advantage of the immobilization of the enzyme in the sedimentable lipid bilayers. Prior to solubilization

TABLE I. Summary of Purification of Hen Oviduct Signal Peptidase

Stage	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)
Rough microsomes	240	4,320	ND[a]	ND
Carbonate-treated microsomes	27	313	ND	ND
Solubilized HOSP before dialysis	32	304	18,400	60
Solubilized HOSP after dialysis	29	148	11,400	77
DEAE pool	90	7.5	16,800	2,240
CM pool	45	4.0	10,400	2,600

[a]ND, not determined. Signal peptidase activity is latent in intact microsomes and was assayed only after solubilization with detergent.

by detergent, more than 90% of contaminating proteins are removed by treating the microsomes with ice-cold sodium carbonate at pH 11.5 (Table I). Under these conditions, only integral membrane proteins, including HOSP [4], remain associated with the lipid bilayers [14]. Peptidase activity is released quantitatively from carbonate-treated microsomes by solubilization with 2.5% (w/v) NP-40 (data not shown), approximately 3 g detergent per gram protein. More than 95% of the protein associated with the microsomes is solubilized under these conditions and remains in the supernatant after centrifugation for 1 hr at 105,000g (Table I).

Prior to chromatography of solubilized HOSP on DEAE cellulose, it is necessary to dialyze the sample to a lower ionic strength. Attempts to solubilize the enzyme with NP-40 in buffers of low ionic strength resulted in incomplete solubilization of HOSP activity. Complete solubilization required the presence of at least 50 mM KCl. Therefore, solubilization was accomplished in buffer A, and dialysis was then performed against DEAE column buffer that contained no KCl and only 5 mM $MgCl_2$. A precipitate forms during this step, and approximately 40% of HOSP activity is apparently lost (Table I). The polypeptide profile revealed by SDS-PAGE analysis of HOSP samples before and after dialysis does not change significantly (compare lanes 2 and 3, Fig. 1A) so it does not appear that specific proteins are precipitating. However, the yield of solubilized enzyme from this approach is better than when solubilization is attempted directly with DEAE column buffer containing 2.5% (w/v) NP-40.

After removal of the precipitate by centrifugation, the supernatant is applied to a column of DEAE cellulose equilibrated at pH 8.2. HOSP activity is not retained by the column and elutes as a broad peak at the column void volume along with the endogenous membrane phospholipid. Significantly, 95% of the applied protein remains bound to the column, but the recovery of HOSP activity appears to be quantitative (Table I). In fact, calculations of enzyme activity at this stage repeatedly show an apparent yield of total HOSP activity in excess of 100%. This result is unexplained but may be due to the inherent inaccuracy of the assay to quantify HOSP activity (see below). Chromatography on DEAE cellulose provides a 30-fold purification of peptidase activity and significantly reduces the number of polypeptides present in active preparations (compare lanes 3 and 4, Fig. 1A).

Anion exchange chromatography is followed by cation exchange on CM cellulose. The pool of HOSP obtained from DEAE chromatography is dialyzed to pH 5.8 and applied to a column of CM cellulose equilibrated at that pH. All of the protein binds to the column under these conditions, but endogenous phospholipid is not

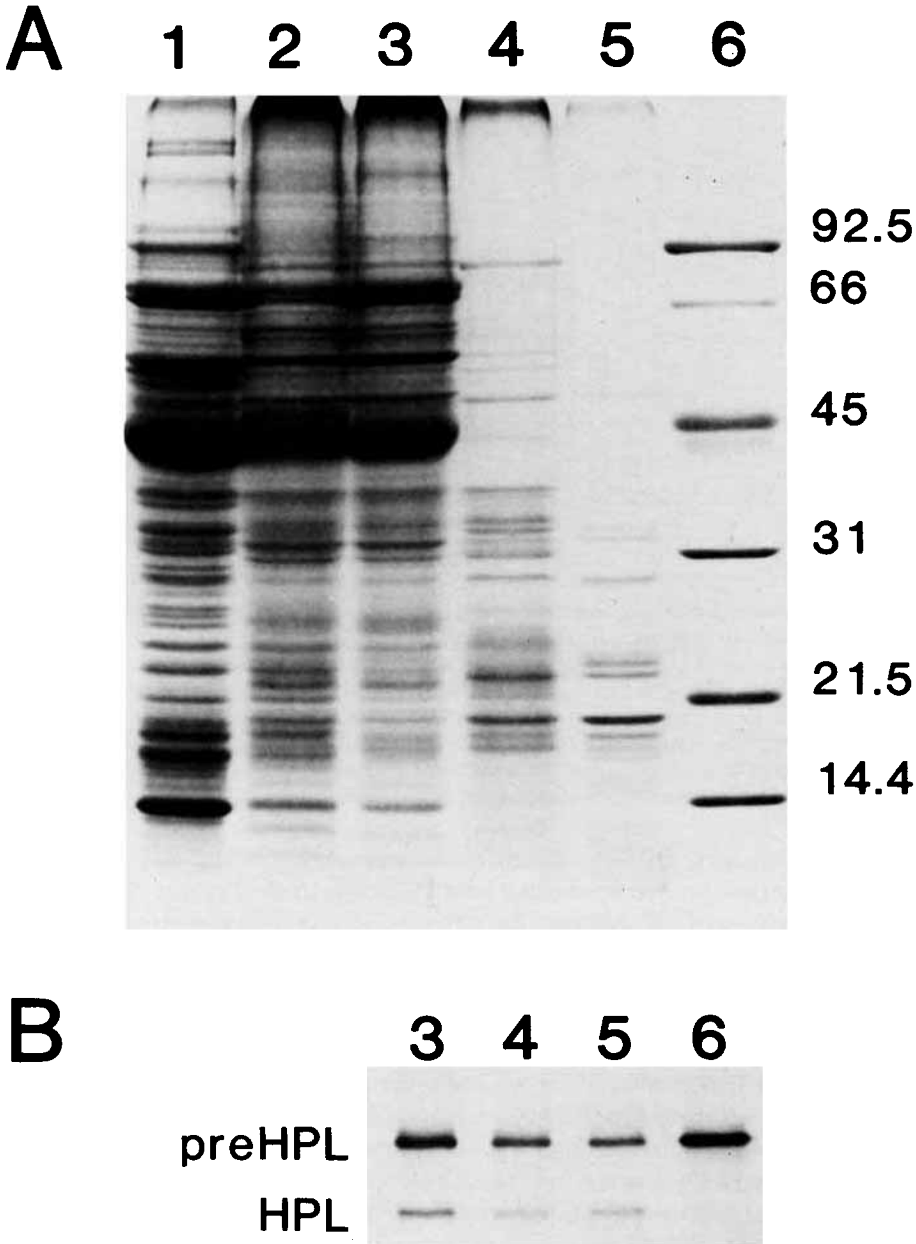

Fig. 1. Polypeptide content and enzyme activity at each stage of purification of hen oviduct signal peptidase. A) Aliquots of HOSP at each stage of purification were separated by SDS-PAGE in a 10–15% linear acrylamide gradient gel and visualized by staining with Coomassie brilliant blue. Lanes: 1, hen oviduct rough microsomes, 100 μg protein; 2, carbonate-treated microsomes, 100 μg protein; 3, solubilized HOSP after dialysis, 100 μg protein; 4, DEAE cellulose pool, 50 μg protein; 5, CM cellulose pool, 50 μg protein; 6, molecular mass standards: phosphorylase b (92.5 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD). B) Autoradiogram of SDS-PAGE gel of reaction mixtures from assays for HOSP activity. Aliquots of [^{35}S]Met-preHPL (5 μl), prepared by cell-free protein synthesis, were incubated with aliquots of HOSP at each stage of the purification for 60 min at 26°C. The reaction products were immunoprecipitated as described in Methods and separated by SDS-PAGE, and the dried gel was exposed to X-ray film for 72 hr. The lanes correspond to the same numbered lanes in A. Lanes: 3, 10 μl detergent-solubilized HOSP; 4, 20 μl DEAE pool; 5, 20 μl CM pool with 2 mg/ml PC; and 6, [^{35}S]Met-preHPL without added HOSP.

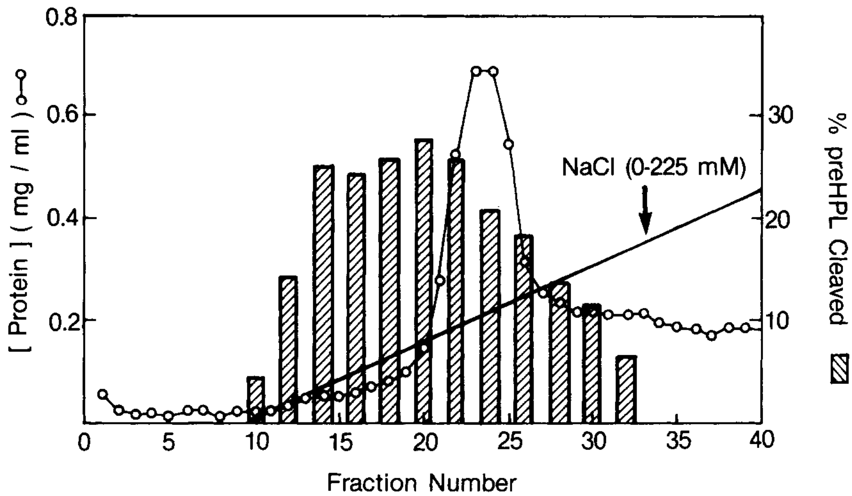

Fig. 2. Chromatography of hen oviduct signal peptidase on carboxymethyl cellulose. The pool of HOSP activity (90 ml) obtained from chromatography on DEAE cellulose was dialyzed to pH 5.8 and applied to a column of carboxymethyl cellulose as described in Methods. After washing with 100 ml of CM buffer, the enzyme is eluted by a gradient of increasing NaCl. Only the fractions (5 ml) obtained during elution of the enzyme are shown. Aliquots (50 μl) of each fraction were assayed for cleavage of [^{35}S]Met-preHPL, and HOSP activity is reported as the percentage of preHPL converted to HPL during a 60-min incubation at 26°C as determined by SDS-PAGE, autoradiography, and densitometry. Protein concentrations were estimated by a modification of the Lowry method [15].

retained. Delipidated HOSP is eluted by an increasing gradient of NaCl (Fig. 2). While the increase in specific enzyme activity is only 1.6-fold (Table I) as a result of chromatography on CM cellulose, the polypeptide profile is improved significantly (compare lanes 4 and 5, Fig. 1A). Fewer than 12 major protein bands are visualized by staining with Coomassie blue. The most prominent band is seen at M_r 19,000, and others range from M_r 54,000 to less than 11,000. We estimate that the overall purification is approximately 600-fold from total microsomes, assuming that no enzyme activity is lost during treatment with carbonate and solubilization with NP-40.

Purified HOSP retains its requirement for a phospholipid environment. This was shown by performing the CM chromatography step without phosphatidylcholine in the column buffer. The CM pool from this experiment was assayed in the presence of a range of concentrations of phosphatidylcholine, and optimal reactivation was observed at 2.0 mg/ml phosphatidylcholine (Fig. 3). The requirement of signal peptidase for phospholipid was first shown with crude, solubilized dog pancreas signal peptidase that was reactivated maximally by phosphatidylcholine [7]. The purified dog peptidase retains this requirement [8].

DISCUSSION

HOSP has been purified more than 600-fold from total oviduct microsomes. Analysis of the partially purified protease by SDS-PAGE reveals approximately 12 major proteins ranging from M_r 54,000 to M_r 11,000 (Fig. 1A). Although this may appear to be far from complete purity, it is revealing to compare these proteins with those of dog pancreas signal peptidase which has recently been purified as a complex

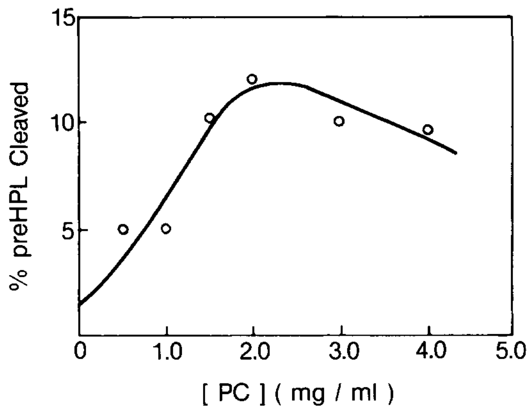

Fig. 3. Reactivation of HOSP by phosphatidylcholine. The pool of HOSP activity obtained from chromatography on DEAE cellulose was eluted from a CM cellulose column as described in Figure 2 but without the addition of exogenous phosphatidycholine. Aliquots (50 μl) of the pool of HOSP were assayed for cleavage of [^{35}S]Met-preHPL in the presence of the indicated concentrations of egg phosphatidylcholine. HOSP activity is reported as the percentage of [^{35}S]Met-preHPL converted to HPL during a 60-min incubation at 26°C as determined by SDS-PAGE, autoradiography, and densitometry.

of six polypeptides with molecular masses of 25, 23, 22, 21, 18, and 12 kD [8]. These six polypeptides copurify through several steps of chromatography and sediment as a complex during sucrose density gradient centrifugation. The 23- and 22-kD bands are glycosylated. Major polypeptides in partially purified HOSP (Fig. 1, lane 5) have molecular masses of 24, 23, 22, 19, 18, and 13 kD. The number, grouping, and relative staining intensities of the protein bands in these two preparations are very similar. It is interesting to note that the most intense Coomassie blue-stained band of dog pancreas signal peptidase [8] was a 21-kD polypeptide and that the most intensive band in HOSP CM pool was at 19 kD (Fig. 1A, lane 5). Furthermore, preliminary data (not shown) indicate that the 23- and 22-kD HOSP bands are stained by the periodic acid–Schiff glycoprotein stain [21]. The earlier observation that HOSP activity seemed to correlate with a 33-kD band [22] is not supported by these results. The results are consistent with the observations made with the dog pancreas enzyme and suggest that HOSP may be part of a similar complex.

The signal peptidase assay based on cleavage of fully synthesized precursor proteins remains a major obstacle to studies of this enzyme. Quantitative data such as those reported in Table I must be interpreted with care, because it is difficult to define a true unit of enzyme activity. The substrates are prepared by cell-free protein synthesis, and only a few hundred femtomoles (10^{-15} mole) can be obtained in a reaction. Therefore, signal peptidase assays are usually conducted in the presence of excess enzyme, up to 3,000 times more HOSP in some cases. The extent of cleavage observed during a 60-min incubation as a function of enzyme concentration is linear up to about 20% conversion of substrate to product. Longer incubations do not increase this yield. In fact, cleavage of preHPL is rarely greater than 50% in spite of the large excess of enzyme.

Uncleaved substrate is resistant to cleavage by freshly added enzyme [9], so it appears that only a small percentage of the substrate is available to the peptidase. The observations that antibody-bound preHPL is a better substrate for HOSP [9] and that processing of preprolactin by dog pancreas signal peptidase is improved if the substrate is pretreated with SDS [8] suggest that the conformation of the substrate plays an important role in recognition by the enzyme. An additional factor that must be considered is the environment of the enzyme. The relative amounts of phospholipid, detergent, and protein present in an assay must be balanced for optimal activity [7,9]. Taken together, these factors make it difficult to compare the activity of the enzyme at different stages of purification and to accurately define a truly quantitative unit of activity.

Further purification of HOSP will determine if the active site of the enzyme is associated with other polypeptide chains that may participate in the transport of proteins into the lumen of the endoplasmic reticulum. With purified HOSP available, studies can be designed to characterize the properties of this enzyme. Small synthetic substrates and inhibitors can be tested for reactivity with the peptidase under well-defined conditions. These reagents will be used to further our understanding of the role of signal peptidase in the translocation process.

ACKNOWLEDGMENTS

These studies were supported by National Institutes of Health grant GM-32861.

REFERENCES

1. Blobel G, Dobberstein B: J Cell Biol 67:852, 1975.
2. Sabatini DD, Kreibich G, Morimoto T, Adesnick M: J Cell Biol 92:1, 1982.
3. Jackson RC, Blobel G: Proc Natl Acad Sci USA 74:5598, 1977.
4. Lively MO, Walsh KA: J Biol Chem 258:9488, 1983.
5. Molllay C, Vilas U, Kreil G: Proc Natl Acad Sci USA 79:2260, 1982.
6. Fujimoto Y, Watanable Y, Uchida M, Ozaki M: J Biochem 96:1125, 1984.
7. Jackson RC, White WR: J Biol Chem 256:2545, 1981.
8. Evans EA, Gilmore R, Blobel G: Proc Natl Acad Sci USA 83:581, 1986.
9. Lively MO, Walsh KA: In Oxender DL (ed): "Protein Transport and Secretion." New York: Alan R. Liss, 1984, pp 379–387.
10. Wolfe PB, Wickner W, Goodman JM: J Biol Chem 258:12073, 1983.
11. Innis MA, Tokunaga M, Williams ME, Loranger JM, Chang S-Y, Chang S, Wu HC: Proc Natl Acad Sci USA 81:3708, 1984.
12. Dev IK, Ray PH: J Biol Chem 259:11114, 1984.
13. Aitken RNC: In Bell DJ, Freeman BM (eds): "Physiology and Biochemistry of the Domestic Fowl." London: Academic Press, 1971, pp 1237–1289.
14. Fujiki Y, Hubbard AL, Fowler S, Lazarow PB: J Cell Biol 93:97, 1982.
15. Peterson GL: Anal Biochem 83:346, 1977.
16. Laemmli UK: Nature 227:680, 1970.
17. Jackson RC: Methods Enzymol 96:784, 1983.
18. Palmiter RD: J Biol Chem 248:2095, 1973.
19. Szczesna E, Boime I: Proc Natl Acad Sci USA 73:1179, 1976.
20. Wyburn GM, Johnston HS, Draper MH, Davidson MF: Q J Exp Physiol 55:213, 1970.
21. Zacharius RM, Zell TE, Morrison JH, Woodlock JJ: Anal Biochem 30:148, 1969.
22. Lively MO, Baker RK: J Cell Biochem 10A:238, 1986.