Biochem. J. (1992) 283, 63–68 (Printed in Great Britain)

Rapid purification of the gastric H^+/K^+-ATPase complex by tomato-lectin affinity chromatography

Judy M. CALLAGHAN,* Ban-Hock TOH,* Richard J. SIMPSON,† Graham S. BALDWIN‡ and Paul A. GLEESON*§

*Department of Pathology and Immunology, Monash University Medical School, Prahran, Victoria 3181, Australia, †Joint Protein Structure Laboratory and ‡Ludwig Institute for Cancer Research (Melbourne Tumour Biology Branch) and The Walter and Eliza Hall Institute for Medical Research, P.O. Royal Melbourne Hospital, Victoria 3050, Australia

We have previously shown that tomato lectin binds specifically to the 60–90 kDa membrane glycoprotein of parietal cell tubulovesicles, the β-subunit of the gastric H^+/K^+-ATPase (proton pump) [Callaghan, Toh, Pettitt, Humphris & Gleeson (1990) J. Cell Sci. **95**, 563–576; Toh, Gleeson, Simpson, Moritz, Callaghan, Goldkorn, Jones, Martinelli, Mu, Humphris, Pettitt, Mori, Masuda, Sobieszczuk, Weinstock, Mantamadiotis & Baldwin (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6418–6422]. Here we have exploited this interaction for the development of a rapid single-step chromatography procedure for the purification of an active pig gastric proton pump complex. Initially, H^+/K^+-ATPase-enriched membranes, prepared from pig gastric microsomes by density-gradient centrifugation, were extracted in 1% Triton X-100 and passed through a 1 ml tomato lectin–Sepharose 4B column. The bound material, eluted with 20 mM-chitotriose, showed a major band with an apparent molecular mass of 95 kDa, and a faint broad band of 60–90 kDa, by SDS/PAGE. *N*-Glycanase treatment of the bound material resulted in the appearance of a 35 kDa band, the size of the protein core of the 60–90 kDa glycoprotein β-subunit. The two components were identified as the 95 kDa α-subunit and the 60–90 kDa β-subunit of the gastric H^+/K^+-ATPase, by immunoreactivity with monospecific antibodies, and by tryptic peptide sequences of the tomato-lectin-bound material. The β-subunit was present in approximately equimolar amounts to the catalytic α-subunit. Whereas the gastric H^+/K^+-ATPase was not active after solubilization in 1% Triton X-100, solubilization of density-gradient-purified membranes in the non-ionic detergent, $C_{12}E_8$, followed by chromatography of the extract on tomato lectin–Sepharose 4B, resulted in the purification of the gastric H^+/K^+-ATPase complex which exhibited K^+-dependent phosphatase activity. This is the first report of a rapid purification of a partially active solubilized gastric H^+/K^+-ATPase complex.

INTRODUCTION

The ouabain-insensitive gastric H^+/K^+-ATPase (proton pump; EC 3.6.1.3), which catalyses the exchange of H^+ and K^+ ions, is responsible for HCl secretion into the stomach (Ganser & Forte, 1973; Saccomani *et al.*, 1977; Rabon & Reuben, 1990). This H^+/K^+-ATPase is localized to the tubulovesicular and canalicular membranes of the mammalian gastric parietal cell (Smolka *et al.*, 1983). The catalytic α-subunit of the gastric proton pump migrates as a 95–100 kDa component on SDS/PAGE (Rabon *et al.*, 1985; Soumarmon *et al.*, 1986; Takaya *et al.*, 1987; Nandi *et al.*, 1987). This catalytic subunit has a high degree of amino acid sequence identity with related ATPases, notably the Na^+/K^+-ATPase and the Ca^{2+}-ATPase (Shull & Lingrel, 1986; Maeda *et al.*, 1988). Recently, a 60–90 kDa membrane glycoprotein, with a 35 kDa core protein, has been shown to be associated with the catalytic subunit of the gastric proton pump (Okamoto *et al.*, 1990; Toh *et al.*, 1990). The amino acid sequence of this associated β-subunit glycoprotein, deduced from cDNA clones obtained from pig (Toh *et al.*, 1990), rabbit (Reuben *et al.*, 1990) and rat (Shull, 1990; Canfield *et al.*, 1990), showed sequence similarity to the β-subunit of Na^+/K^+-ATPase. Both the catalytic α-subunit and this associated β-subunit of the gastric proton pump have been shown to be the major molecular targets of autoantibodies from humans with autoimmune gastritis (Karlsson *et al.*, 1988; Goldkorn *et al.*, 1989; Toh *et al.*, 1990) as well as from mice with experimentally induced autoimmune gastritis (Jones *et al.*, 1991*b*; Gleeson & Toh, 1991).

Although many methods for the preparation of gastric membrane vesicles enriched in the gastric H^+/K^+-ATPase have been published (Forte *et al.*, 1975; Saccomani *et al.*, 1975, 1977; Nandi *et al.*, 1987; Rabon *et al.*, 1988), to date there has been only a single report on the purification of solubilized gastric H^+/K^+-ATPase (Takaya *et al.*, 1987). These authors purified emulgen-solubilized pig H^+/K^+-ATPase by a multistep procedure taking about 4 days. The protein was purified to a single band of 94 kDa on SDS/PAGE; however, the yield of purified material was low (0.156 mg/104 mg of H^+/K^+-ATPase-enriched membranes). In addition, since no other components were detected in this purified preparation, the associated β-subunit glycoprotein was presumably dissociated during the purification procedure.

Previously, we have shown that the polylactosamine-specific tomato lectin specifically binds a 60–90 kDa glycoprotein of the canalicular and tubulovesicular membranes of gastric parietal cells from a number of different species (Callaghan *et al.*, 1990). Subsequently, this 60–90 kDa membrane glycoprotein was identified as the β-subunit of the proton pump (Toh *et al.*, 1990). Since this glycoprotein is the major tomato-lectin-bound component from gastric membranes, we have exploited this interaction for the purification of the pig gastric proton pump α- and β-subunit complex by a single chromatography step. Furthermore, solubilization of density-gradient-purified gastric membranes in the detergent $C_{12}E_8$ allowed the purification of a partially active H^+/K^+-ATPase complex. This method should have general applicability for the rapid isolation of an active gastric proton pump complex from a variety of different species. The purified

Abbreviations used: H^+/K^+-ATPase, H^+/K^+-transporting ATPase; Na^+/K^+-ATPase, Na^+/K^+-transporting ATPase; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.2).

§ To whom correspondence should be addressed.

proton pump complex will be invaluable for studies on the subunit interactions of the active enzyme, for the identification of the autoepitopes recognized by the parietal cell autoantibodies and for the generation of proton-pump-specific auto-reactive T-cell clones from humans and from mice with autoimmune gastritis.

EXPERIMENTAL

Preparation of pig gastric mucosal membranes

The body portions of pig stomachs were washed with phosphate-buffered saline (PBS), and the surface mucus was removed by scraping with a glass slide. The mucosa was dissected from the muscle, cut into fragments and agitated in PBS to remove remaining mucus, and then homogenized for 2 min in a Waring blender in 50 mM-Tris/HCl buffer, pH 7.4, containing 250 mM-sucrose, 25 mM-KCl, 10 mM-$MgSO_4$, and 1 mM-phenylmethanesulphonyl fluoride (PMSF) (60 g of tissue/200 ml of buffer). The resulting homogenate was centrifuged first at 600 *g* for 10 min to remove coarse fragments and nuclei, then at 15000 *g* for 30 min to remove mitochondria, and finally at 100000 *g* for 60 min to obtain the total gastric membrane fraction. The membrane pellet was suspended in 50 mM-Hepes buffer, pH 7.6, containing 2 mM-EDTA and 1 mM-PMSF. All procedures were performed at 4 °C. The protein content of the membrane preparations was determined using the Bio-Rad protein assay (Bradford, 1976) after solubilizing the sample in 1 M-NaOH for 30 min at room temperature. The protein concentration of the preparations was adjusted to 10 mg/ml and the membranes were stored at −70 °C.

Preparation of H^+/K^+-ATPase-enriched gastric membranes

Samples (8 ml) of total gastric membranes, resuspended in the 0.25 M-sucrose homogenizing buffer, were layered over 16 ml of 37% (w/v) sucrose and centrifuged at 75000 *g* for 90 min. The membranes at the sucrose interface were removed, diluted 4-fold in 0.25 M-sucrose buffer, and centrifuged at 100000 *g* for 60 min. The resulting membrane pellet was resuspended in 50 mM-Hepes, pH 7.6, containing 2 mM-EDTA and stored at −70 °C. Membrane preparations were analysed by SDS/PAGE (Laemmli, 1970) after incubation in electrophoresis buffer at room temperature for 15 min to avoid aggregation of the 95 kDa H^+/K^+-ATPase α-subunit (Jones *et al.*, 1991*a*), and the polyacrylamide gels were stained in 0.2% Coomassie Blue R-250.

Membrane fractions were solubilized with 1.0% (w/v) Triton X-100 or with $C_{12}E_8$ (Sigma) at 0.5 mg of $C_{12}E_8$/mg of membrane protein, on ice for 30 min and insoluble residue was removed by centrifugation at 100000 *g* for 30 min. The insoluble residue of the Triton X-100 extraction contained no detectable H^+/K^+-ATPase, as assessed by *p*-nitrophenylphosphatase activity and by SDS/PAGE analysis. The ratio of $C_{12}E_8$/membrane protein was chosen to maximize stable enzyme activity recovered in the soluble fraction. At 0.5 mg of $C_{12}E_8$/mg of membrane protein, approximately equal *p*-nitrophenylphosphatase activity was recovered in the insoluble residue and the soluble fraction. Proteinase inhibitors, PMSF (1 mM), aprotonin (500 kallikrein-inhibitor units/ml), leupeptin (0.5 mg/ml) and pepstatin (1.0 mg/ml), were added to all membrane preparations. PMSF was from Sigma Chemical Co. and aprotinin, leupeptin and pepstatin were from United States Biochemical Corp., Cleveland, OH, U.S.A.

K^+-dependent *p*-nitrophenylphosphatase activity

p-Nitrophenylphosphatase activity of membrane preparations was determined by measuring the *p*-nitrophenol released from the hydrolysis of *p*-nitrophenyl phosphate (Nandi *et al.*, 1987). Assays were carried out in a final volume of 1 ml in 50 mM-Tris/HCl, pH 7.5, containing 2 mM-$MgCl_2$, 5 μmol of *p*-nitrophenyl phosphate and 5–50 μg of protein, in the absence and presence of 25 mM-KCl. Incubations were carried out for 15 min at 37 °C, and the reaction was stopped with 1 ml of 1.5 M-NaOH. The *p*-nitrophenol released was measured spectrophotometrically at 410 nm. The μmol of substrate hydrolysed was calculated on the basis of the molar absorption coefficient of *p*-nitrophenol at 410 nm (1.45×10^4 litre·mol^{-1}·cm^{-1}).

Lectin blotting

Tomato lectin blotting was performed as described by Callaghan *et al.* (1990), except that samples were not heated before separation by SDS/PAGE.

Lectin affinity chromatography

Tomato lectin (Sigma) was coupled to CNBr-activated Sepharose 4B (Pharmacia) at a concentration of 1 mg/ml following the manufacturer's instructions. H^+/K^+-ATPase-enriched membranes were solubilized for 30 min at 4 °C in 1% Triton X-100 in PBS, and a portion containing 600 μg of protein (500 μl) was recycled three times over a 1 ml tomato lectin–Sepharose 4B column. The column was washed extensively with 50 mM-Hepes, pH 7.6, containing 0.1% Triton X-100 and 0.15 M-NaCl and the bound material was eluted with 20 mM-*NN'N''*-triacetylchitotriose (chitotriose) (Sigma Co.) in this buffer or with 0.1 M-glycine/HCl buffer, pH 2.5. Typically 115 μg of protein was recovered in the bound fraction eluted with chitotriose. $C_{12}E_8$ extracts of H^+/K^+-ATPase-enriched membranes were chromatographed in a similar manner except that 1 mg of protein (1.5 ml) was loaded on to the column which was then washed extensively with 50 mM-Hepes, pH 7.6, containing 0.1 mg of $C_{12}E_8$/ml and 0.15 M-NaCl, and the bound material was eluted with 20 mM-chitotriose in this buffer.

Immunoblotting

Membrane preparations, solubilized in 1% (w/v) Triton X-100, and fractions from the lectin column were subjected to SDS/PAGE after reduction with 50 mM-dithiothreitol. The samples were not heated before separation by SDS/PAGE. The separated proteins were electrophoretically transferred to unmodified nitrocellulose membrane (Schleicher and Schuell; 0.45 μm pore size) overnight at 60 V in a Bio-Rad blotting cell, and immunoblotting was performed as described (Callaghan *et al.*, 1990; Jones *et al.*, 1991*a*). The monoclonal antibody 2B6 is specific for the β-subunit of the H^+/K^+-ATPase (Jones *et al.*, 1991*b*) and was used as undiluted supernatant. The rabbit antiserum specific for the 95 kDa catalytic subunit of the rat H^+/K^+-ATPase was raised to a bacterial fusion protein containing the hydrophilic domain of the α-subunit and affinity-purified as described (Jones *et al.*, 1991*a*). Control experiments were carried out in parallel with IgG1 isotype antibody and with normal rabbit serum.

Treatment with *N*-Glycanase (peptide:*N*-glycosidase F; EC 3.2.2.18)

The bound material recovered from the lectin affinity chromatography column was boiled for 3 min in 0.5% SDS containing 0.1 M-2-mercaptoethanol and the solution centrifuged to remove insoluble material. The supernatant was collected and diluted with 1.5 vol. of 0.1 M-Tris/acetate buffer, pH 8.6, containing 10 mM-EDTA and 3.5% (w/v) Nonidet P-40. Portions of the sample were incubated at 37 °C for 16 h with 6.5 units of *N*-glycanase/ml (Genzyme Corp., Boston, MA, U.S.A.) in 0.1 M-Tris/acetate buffer, pH 8.6, containing 10 mM-EDTA (final volume 40 μl). A control incubation in the presence of buffer alone

was included. The preparations were examined by SDS/PAGE followed by silver staining (Ansorge, 1983). The silver-stained bands were quantified by scanning densitometry using an LKB Ultrascan XL densitometer.

Isolation and amino acid sequencing of tryptic peptides

Samples of the tomato-lectin-bound material were precipitated with methanol then reduced, carboxymethylated and digested with trypsin as described previously (Baldwin *et al.*, 1987). Tryptic peptides were purified by multi-dimensional microbore reversed-phase h.p.l.c. (Simpson *et al.*, 1988). Automated Edman degradation of tryptic peptides was performed using Applied Biosystems sequencers (models 470A and 477A) equipped with on-line phenylthiohydantoin amino acid analysers (model 120A). Total phenylthiohydantoin amino acid derivatives from the sequencers were injected on to the liquid chromatographs using a modified sample-transfer device described elsewhere (Begg & Simpson, 1989). Polybrene (Klapper *et al.*, 1978) was used as a carrier.

RESULTS

A pig gastric membrane fraction, prepared from total gastric membranes by density-gradient centrifugation, showed approximately a 10-fold enrichment of ouabain-insensitive K^+-dependent phosphatase activity, an activity associated with the gastric H^+/K^+-ATPase. The activity of the H^+/K^+-ATPase-enriched membranes was 55 μmol/h per mg, an activity comparable with previous reports (Saccomani *et al.*, 1977; Soumarmon *et al.*, 1983; Nandi *et al.*, 1987). In addition, these membranes were also enriched for a Coomassie Blue-staining component of 95 kDa (Fig. 1, lane A), the expected size of the catalytic α-subunit of the gastric H^+/K^+-ATPase. Lectin blotting showed that tomato lectin reacted with a broad 60–90 kDa smear from the pig H^+/K^+-ATPase-enriched membrane preparations (Fig. 1, lane B) consistent with our previous observations (Callaghan *et al.*, 1990). In addition, the mouse monoclonal antibody, 2B6, which recognizes the 35 kDa protein core of the 60–90 kDa proton pump β-subunit, immunoblots the 60–90 kDa glycoprotein from pig H^+/K^+-ATPase-enriched membranes (Fig. 2).

H^+/K^+-ATPase-enriched membranes were used for the purification of the H^+/K^+-ATPase, in preference to crude gastric membranes, to avoid contamination by soluble mucin, which is also bound by tomato lectin (Callaghan *et al.*, 1990). Chromatography of 1% Triton X-100 H^+/K^+-ATPase-enriched membrane extracts on tomato lectin–Sepharose 4B resulted in all the 60–90 kDa glycoprotein being eluted in the bound fraction, as detected by immunoreactivity with the monoclonal antibody 2B6 (Fig. 2). The 95 kDa α-subunit of the H^+/K^+-ATPase was also eluted exclusively in the bound fraction from the tomato lectin column, as detected by immunoreactivity with a rabbit antiserum raised to a bacterial fusion protein containing the hydrophilic region of the proton pump α-subunit (Fig. 2). The bound fraction from the lectin column showed a single major silver-stained component of 95 kDa (Fig. 3, lane 1); in contrast, the 60–90 kDa region of the gel stained only weakly with the silver stain. No additional components were detected on the silver-stained gel. Typically, 115 μg of purified material was recovered from 600 μg of protein extracted from density-gradient-purified membrane. Therefore, on the basis of the recovered protein, the purified material represents a 5-fold enrichment from the membrane preparation prepared by density-gradient centrifugation and a 50-fold enrichment from the crude gastric membrane preparation. The recovery of bound material from the tomato

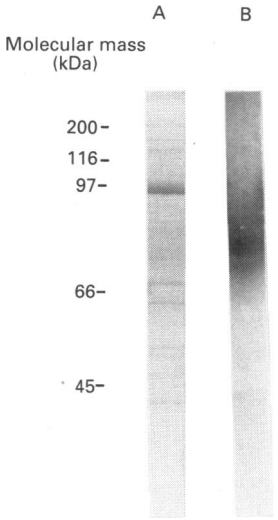

Fig. 1. SDS/PAGE analysis and tomato lectin blotting of pig H^+/K^+-ATPase-enriched membrane preparation

Lane A, Coomassie Blue-stained polyacrylamide gel (7.5%) and lane, B tomato lectin blotting of pig H^+/K^+-ATPase membrane preparation. In lane B, proteins (15 μg) of the membrane preparation were separated by SDS/PAGE and transferred to nitrocellulose. The nitrocellulose was blocked with 3% casein/PBS, cut into strips, incubated with 50 μg of biotinylated tomato lectin/ml, washed and incubated with streptavidin–biotinylated peroxidase complex. Bound peroxidase was detected by incubation with 0.05% diaminobenzidine in PBS containing 0.03% $NiCl_2$ and 0.02% H_2O_2. A control in the presence of 10 mM-chitotriose showed very little staining.

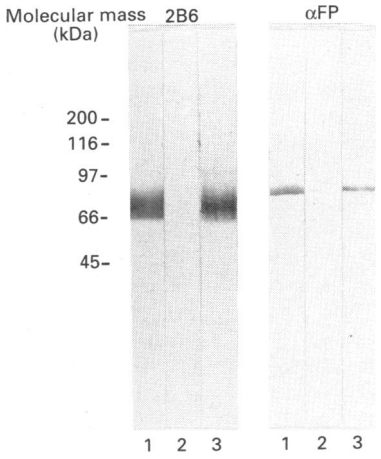

Fig. 2. Immunoblot analysis of fractions of solubilized pig gastric membranes from tomato lectin column

Immunoblot analysis of the H^+/K^+-ATPase-enriched membrane extract (lanes 1), and the unbound (lanes 2) and bound (lanes 3) fractions from the tomato lectin column, with monoclonal antibody 2B6 (2B6), specific for the 60–90 kDa β-subunit glycoprotein, and an affinity-purified rabbit antiserum to the 95 kDa catalytic subunit of the H^+/K^+-ATPase (αFP). Equal amounts of each fraction were analysed after incubation in SDS sample buffer at room temperature. Proteins in the fractions were separated on a 7.5% (w/v) polyacrylamide gel under reducing conditions before transfer to nitrocellulose membrane. The membrane was blocked, cut into strips, and incubated with either monoclonal antibody 2B6 or an affinity purified rabbit anti-(fusion protein) serum (diluted 1:20 in blocking solution) followed by either rabbit anti-(mouse Ig) antibody–horseradish peroxidase or sheep anti-(rabbit Ig antibody)–horseradish peroxidase (diluted 1:100) respectively. Bound peroxidase was detected with diaminobenzidine/H_2O_2.

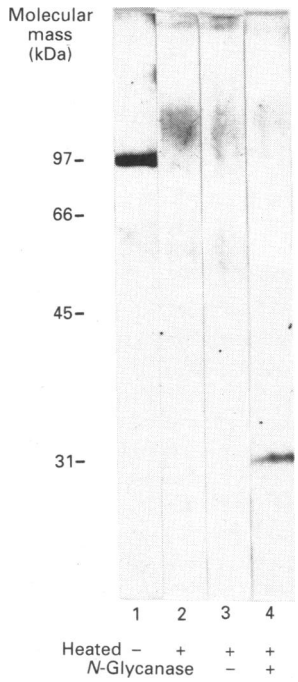

Fig. 3. SDS/PAGE analysis of tomato-lectin-bound material from solubilized pig H^+/K^+-ATPase-enriched membrane preparations

Silver-stained gel of bound material from the tomato lectin column. Untreated samples were incubated either at room temperature (lane 1) or 100 °C (lane 2) before SDS/PAGE. Samples were denatured by heating at 100 °C in SDS, as described in the Experimental section, and were incubated at 37 °C for 20 h in the absence (lane 3) or presence (lane 4) of 6.5 units of *N*-Glycanase/ml, before analysis of SDS/PAGE. Samples were separated on a 10% (w/v) polyacrylamide gel under reducing conditions.

lectin column by elution with 20 mM-chitotriose was 3-fold higher than by elution with a low-pH buffer.

Digestion of the bound fraction from the tomato lectin column with *N*-Glycanase resulted in the appearance of a sharp band at 35 kDa (Fig. 3, lane 4), the size of the protein core of the 60–90 kDa glycoprotein. The carbohydrate of the 60–90 kDa glycoprotein therefore appears to decrease the intensity of silver staining. Under the conditions required for *N*-Glycanase digestion, the 95 kDa component aggregates and migrates as higher-molecular-mass complexes (Jones *et al.*, 1991*a*). The SDS/PAGE gels were scanned and the ratio of the two components was determined by comparing the stained 95 kDa band in the untreated sample (Fig. 3, lane 1) with that of the 35 kDa band in the *N*-Glycanase-treated sample (Fig. 3, lane 4). The relative staining of the 95 kDa α-subunit to the 35 kDa protein core of the β-subunit was approximately 2.5:1. Allowing for the difference in molecular masses, calculated from the predicted amino acid structure, and assuming an equivalent intrinsic staining, the α- and β-subunits of the gastric H^+/K^+-ATPase co-purified from the tomato lectin column were present in a molar ratio of approximately 1:1.3. The observed molar ratio of the two subunits is consistent with that reported for the complex isolated by either immunoaffinity chromatography with a monoclonal antibody to the β-subunit (Jones *et al.*, 1991*a*) or wheat germ agglutinin chromatography (Okamoto *et al.*, 1990).

The tomato-lectin-bound fraction was digested with trypsin and the tryptic digest separated by multidimensional h.p.l.c. Nine tryptic peptides were sequenced; of these, three peptides have sequences identical with the pig H^+/K^+-ATPase α-subunit (Table 1), while three other peptides are derived from the

Table 1. Sequences of the tryptic peptides obtained from tomato-lectin-bound fraction

Samples of the tomato-lectin-bound material were reduced, carboxymethylated and digested with trypsin. Tryptic peptides were purified by multidimensional, microbore reversed-phase h.p.l.c., and sequenced on a gas-phase sequencer. The yields of peptides ranged from 40 to 100 pmol. Lower-case letters represent uncertain amino acid assignments. Numbers refer to the position of the *N*-terminal residue of the tryptic peptides to the sequence of the α-subunit (Maeda *et al.*, 1988) and β-subunit (Toh *et al.*, 1990) of the pig gastric H^+/K^+ ATPase.

Peptide no.	Subunit	Position	Sequence
1	α	173	NLVPQQATVI
2	α	777	LIFDNLK
3	α	1022	CCPGSWWDQELY
4	β	65	tIDPYTPDYQDqLK
5	β	225	SLHYFPYYGK
6	β	236	AQPHYSNPLVAAK
7			YVMGLHFWDRLYG
8			NRKLLDIas
9			ATVPDYPVLK

60–90 kDa glycoprotein (Toh *et al.*, 1990) previously identified as the β-subunit of the H^+/K^+-ATPase. These amino acid sequence data therefore confirm that the components purified by tomato lectin chromatography are the α- and β-subunit complex of the gastric H^+/K^+-ATPase. The yields of α- and β-subunit tryptic peptides recovered by h.p.l.c. are consistent with the two subunits present in approximately equimolar proportions. Three additional tryptic peptide sequences were obtained which did not correspond to either the α- or β-subunit of the H^+/K^+-ATPase (Table 1). It is possible that these peptides are derived from an additional subunit of the H^+/K^+-ATPase.

The conditions of solubilization and chromatography were initially chosen to maximize purity of the proton pump complex. H^+/K^+-ATPase-associated phosphatase activity was not detected in our Triton X-100 extracts. As the non-ionic polyoxyethylene detergent, $C_{12}E_8$, has successfully been employed to purify a solubilized active Na^+/K^+-ATPase (Esmann, 1988), we examined the ability of this detergent to allow the solubilization and purification of an active gastric H^+/K^+-ATPase. Extraction of the H^+/K^+-ATPase-enriched membranes with $C_{12}E_8$ (0.5 mg of detergent/mg of protein) resulted in a soluble H^+/K^+-ATPase with a K^+-dependent *p*-nitrophenylphosphatase activity of 3.4 μmol/h per mg which was stable for at least 8 h at 4 °C. Furthermore, purification of the $C_{12}E_8$-solubilized gastric H^+/K^+-ATPase on tomato lectin–Sepharose 4B resulted in an active preparation, with a K^+-dependent phosphatase activity of approximately 6 μmol/h per mg. SDS/PAGE analysis of the $C_{12}E_8$-solubilized tomato-lectin-purified H^+/K^+-ATPase was similar to the material purified in Triton X-100 (not shown).

DISCUSSION

We have previously shown that tomato lectin binds to the heavily *N*-glycosylated β-subunit of the proton pump of the tubulovesicular and canalicular membranes of parietal cells from a number of different species. No binding of tomato lectin to other cell membranes of the gastric mucosa was observed. The selective interaction of tomato lectin with the β-subunit of the proton pump suggests that the β-subunit contain glycans with polylactosamine sequences (Merkle & Cummings, 1987). Here we have developed a rapid single-step procedure for the puri-

fication of the detergent-solubilized pig gastric proton pump (H^+/K^+-ATPase) by tomato lectin affinity chromatography.

The material isolated from the tomato lectin column showed a major band of 95 kDa on SDS/PAGE, which was identified as the α-subunit of the H^+/K^+-ATPase on the basis of (1) molecular mass, (2) immunoreactivity with an antiserum specific for the α-subunit of the H^+/K^+-ATPase and (3) identity of amino acid sequences of three tryptic peptides isolated from the tomato-lectin-bound fraction with the known sequence of the pig H^+/K^+-ATPase catalytic subunit. A recent report suggests that the rat α-subunit of the proton pump is glycosylated with an *N*-glycan chain (Tai *et al.*, 1989); however, as this carbohydrate is susceptible to cleavage by endoglycosidase H, it is probably a high-mannose *N*-glycan which would not be expected to interact with tomato lectin. Therefore the α-subunit does not appear to interact directly with tomato lectin but rather is copurified with the β-subunit glycoprotein. We have observed a similar co-purification of the two subunits using a monoclonal antibody specific for the dog H^+/K^+-ATPase β-subunit (Jones *et al.*, 1991*a*). The 60–90 kDa β-subunit from the tomato lectin column stained poorly with protein stains, but was readily detected by immunoblotting using specific antibodies. Further, the 35 kDa protein core of the 60–90 kDa β-subunit was visualized on polyacrylamide gels by silver staining after removal of the carbohydrate with *N*-Glycanase. The two subunits of the H^+/K^+-ATPase are present in the purified complex in approximately equimolar proportions, as determined by silver staining of polyacrylamide gels and the yields of recovered tryptic peptides. Although silver-stained polyacrylamide gels indicated that the α- and β-subunits were the only components in the tomato-lectin-bound fraction, tryptic peptide sequences indicated the presence of an additional component. Therefore the gastric H^+/K^+-ATPase may consist of more than two subunits. Baldwin (1990) has presented evidence for the existence of a second, minor, population of the β-subunit of the H^+/K^+-ATPase, with no accompanying α-subunit, in a heavy fraction of pig gastric mucosal membranes.

Takaya *et al.* (1987) purified the gastric proton pump from emulgen-solubilized pig gastric membranes by a multi-step procedure. However, the only component detected in their preparation was the 94 kDa α-subunit, suggesting that the associated β-subunit was lost during the purification procedure. The method reported here, using tomato lectin to purify the proton pump complex, has the following advantages over the previous method of Takaya *et al.* (1987): (1) the purified material consists of both the α- and β-subunits; (2) the yield of purified material is about 80 times that obtained by Takaya *et al.* (1987) (on the basis of density-gradient-purified membranes as the starting material); (3) the purification method by tomato lectin chromatography is very rapid, and can be completed within hours, whereas the earlier method involved a number of chromatographic steps and required about 4 days.

Okamoto *et al.* (1990) fractionated detergent-solubilized pig membrane fractions by lectin affinity chromatography using wheat germ agglutinin and *Ricinus communis* I lectin. While the α- and β-subunit complex of the proton pump was recovered in the bound fraction from the wheat germ agglutinin column, a number of additional glycoproteins were also recovered in this fraction. Therefore tomato lectin appears to provide a more specific lectin support for purification of the gastric proton pump from membrane extracts.

An active gastric H^+/K^+-ATPase has been prepared in membrane-bound form from the gastric mucosa of a variety of species by glycerol- and/or sucrose-density-gradient centrifugation (Saccomani *et al.*, 1975, 1977; Forte *et al.*, 1975; Nandi *et al.*, 1987; Rabon *et al.*, 1988). These membrane preparations showed an enrichment of the 95 kDa catalytic subunit. However, further purification of an active gastric proton pump has proved difficult, largely because of problems associated with loss of enzyme activity on detergent solubilization and on storage. This instability may be due to the requirement of an oligomeric structure of the H^+/K^+-ATPase for functional activity (Rabon & Reuben, 1990). To date, a highly active pure H^+/K^+-ATPase has not been obtained. When gastric membranes were solubilized in the non-ionic detergent $C_{12}E_8$, the purified proton pump from the tomato lectin support showed a K^+-dependent phosphatase activity of 6 μmol/h per mg; this represents a 2-fold increase in specific activity compared with the solubilized membranes. However, the specific activity of the purified enzyme is considerably lower than the specific activity of the density-gradient-purified membrane fraction. The pig gastric H^+/K^+-ATPase purified by Takaya *et al.* (1987) also had only a very low specific *p*-nitrophenylphosphatase activity (4.86 μmol/h per mg). Again, this problem of low specific activity of the solubilized enzyme probably reflects the structural requirements of the proton pump for activity.

The method reported here should have general applicability for the rapid isolation of an active gastric proton pump from a variety of different species. The isolation of the gastric proton pump complex will be valuable for studies investigating the subunit composition of the active enzyme and for further studies directed towards the isolation of a fully active soluble enzyme. In addition, we have recently shown that the proton pump is a major target for immune recognition by autoantibodies from patients with autoimmune gastritis and pernicious anaemia, as well as by autoantibodies from experimentally induced autoimmune gastritis in mice (Toh *et al.*, 1990; Jones *et al.*, 1991*b*; Gleeson & Toh, 1991). The purified proton pump complex will therefore be invaluable for the identification of the autoepitopes recognized by these antibodies, and will allow the development of a specific immunoassay for the rapid detection of these autoantibodies and facilitate the generation of proton-pump-specific auto-reactive T-cell clones from humans and from mice with autoimmune gastritis.

We thank Dr. Ian van Driel for many stimulating discussions. This work was supported in part (P.A.G. and B.-H.T.) by grants from the National Health and Medical Research Council of Australia.

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Received 1 July 1991/29 August 1991; accepted 25 September 1991