Rapid purification and direct microassay of calbindin$_{9kDa}$ utilizing its solubility in perchloric acid

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The 9 kDa calcium-binding protein, calbindin$_{9kDa}$, was found to be soluble in 7% (v/v) perchloric acid. Calbindin$_{9kDa}$ was easily purified from rat duodenum in 1 day with perchloric acid precipitation followed by reverse-phase h.p.l.c. The yield was 21.4 ± 2.3 nmol/g wet weight of tissue (mean $\pm$ S.E.M.; $n = 3$) from normally fed 7–8-week-old rats (approx. 70% recovery). The purification was also effective with rabbit duodenum calbindin$_{9kDa}$, but not with various other EF-hand calcium-binding proteins tested in the rat. Several criteria (h.p.l.c., u.v. spectrum, denaturing two-dimensional PAGE, N-terminal sequencing) indicated that the rat calbindin$_{9kDa}$ was purified to homogeneity and was not affected by proteolysis. High-affinity calcium-binding properties were retained and no evidence of isoforms or charge modification was observed. Residue 59, identified as Asn (not Asp as previously reported), was fully amidated. When adopted as a microassay with isocratic h.p.l.c., the perchloric acid procedure enabled rapid (less than 6 min) and direct (peptide bond absorbance) quantification of less than 1 pmol of calbindin$_{9kDa}$. This new approach to purification and assay will be of particular utility for investigations of calbindin$_{9kDa}$ in previously intractable low-abundance sources (e.g. cultured cells).

INTRODUCTION

The 9 kDa calcium-binding protein, calbindin$_{9kDa}$, contains two high-affinity sites (so-called EF-hands; $K_{app.}$ approx. 0.5 μM Ca^{2+} in physiological conditions) and is the smallest known member of the calmodulin superfamily. In mammalian duodenum, where it was discovered, calbindin$_{9kDa}$ is abundant ($\sim$ 1% of soluble protein) and its vitamin D_3-dependent expression parallels transcellular calcium transport (reviewed in [1,2]). Other tissues expressing significant levels of this highly conserved cytosolic protein include placenta and yolk sac, uterus and fallopian tube, kidney, lung, cartilage and bone. In addition to regulation by vitamin D_3, tissue-specific effects of dexamethasone, oestrogen and progesterone have been identified [3,4]. More than 25 years of research have failed to reveal the molecular function of calbindin$_{9kDa}$, although in all locations an association of calbindin$_{9kDa}$ expression with calcium transport seems likely [1,2].

Calbindin$_{9kDa}$ is a particularly robust molecule when the calcium sites are occupied, and its resistance to heat denaturation has long been utilized as an efficient purification step. N.m.r. studies showed that the calbindin$_{9kDa}$ structure is barely affected by temperatures up to 90 °C, alkali up to pH 14, or 8 M urea [5,6]. With the exception of its exposed N-terminus, calcium-loaded calbindin$_{9kDa}$ is resistant to proteolysis [7,8].

This paper describes another manifestation of calbindin$_{9kDa}$ stability, i.e. solubility in perchloric acid. This unusual property was exploited in the development of powerful new purification and microassay procedures, and the calbindin$_{9kDa}$ isolated from rat duodenum was characterized.

EXPERIMENTAL

Materials

Analytical-grade 70% (v/v) perchloric acid was obtained from Riedel de Haen AG (Hannover, Germany). Electrophoresis reagents (Electran grade) were purchased from BDH (Poole, Dorset, U.K.) and $^{45}CaCl_2$ was from Amersham. Bovine erythrocyte ubiquitin and sequencing-grade trypsin were products of Sigma (St. Louis, MO, U.S.A.). Trifluoroacetic acid (Sequanal grade) was supplied by Pierce (Rockford, IL, U.S.A.), and h.p.l.c.-grade acetonitrile by Baker (Phillipsburg, NJ, U.S.A.). Cytosol was prepared from rat brain and skeletal muscle as described below for duodenum, except that homogenization was achieved with a motorized tissue disperser (Tissue Tearor; Biospec Products).

Purification of calbindin$_{9kDa}$

Mixed-sex 7–8-week-old Wistar rats were fed on a standard pellet chow *ad libitum*. Unless indicated otherwise, the following operations were done at 2–4 °C. Duodenal mucosa scraped from the proximal 10 cm of intestine was dispersed by hand in 1.5 vol. of 10 mM Tris/HCl, pH 7.5 (at 4 °C), 1 mM dithiothreitol and 0.5 mM phenylmethanesulphonyl fluoride using a glass–Teflon homogenizer. After centrifuging at 15000 *g* for 10 min in an Eppendorf microcentrifuge, the supernatant (referred to as cytosol) was vortex-mixed with one-tenth volume 70% perchloric acid and placed on ice for 5 min. Where indicated, the supernatant was instead heated in a 100 °C water bath for 5 min. After centrifuging (5 min, 15000 *g*) the acid-treated supernatant was made 10% (v/v) in acetonitrile, centrifuged (17000 *g* for 2 min in an IEC microcentrifuge) and passed through a 0.22 μm pore-size membrane filter (Ultrafree-MC Durapore; Millipore). Heat-treated supernatant was made 0.2% (v/v) in trifluoroacetic acid and then processed in the same manner. Samples were applied to a reverse-phase h.p.l.c. (r.p.-h.p.l.c.) column equilibrated with 0.1% (v/v) trifluoroacetic acid and 10% (v/v) acetonitrile. Wide-pore (30 nm) silica-based butyl (GLT; from SGE) and octyl (Aquapore; from ABI) columns were used under the conditions detailed in the legend to Figure 3. The yields given in the Results section were calculated from integrated peak areas,

Abbreviation used: r.p.-h.p.l.c., reverse-phase h.p.l.c.
The protein sequence data reported here were deposited in the PIR database (NBRF, U.S.A.); accession number A37336.

calibrated with a calbindin$_{9kDa}$ standard run on the same day. H.p.l.c.-purified calbindin$_{9kDa}$ was dried in a vacuum centrifuge, resuspended in 10 mM Tris/HCl, pH 7.5, quantified by spectral analysis ($A^{1\%}_{1cm,280}$ 3.3 [9]), and stored at −80 °C. When indicated by the spectral data (see the Results section), the sample was diluted with 1 vol. of 2 mM EGTA (pH 7.5), applied to an anion-exchange f.p.l.c. column (PL-SAX; from Polymer Labs) equilibrated at 1 ml/min in 5 mM Tris/HCl, pH 7.5, and 1 mM EGTA, and eluted with a linear gradient to 0.5 M NaCl over 30 min. All h.p.l.c. and f.p.l.c. was done at room temperature.

Rabbit calbindin$_{9kDa}$ was purified using perchloric acid precipitation and r.p.-h.p.l.c. as described above for the rat protein. The quantification given in the Results section is for three small-scale purifications from a single cytosol preparation made with the proximal 30 cm of duodenum from a 3-month-old male New Zealand White rabbit, calibrated with a rat calbindin$_{9kDa}$ standard.

Gel electrophoresis procedures

SDS/PAGE was carried out on 0.75 mm-thick slab gels with a Tricine discontinuous buffer system [10]. Sample loading buffer contained 2 mM EGTA to overcome the differential mobility exhibited by calbindin$_{9kDa}$ in the presence of calcium [11]. Denaturing two-dimensional PAGE was essentially as described [12]. Removal of Ampholytes, which co-migrated in the second dimension with calbindin$_{9kDa}$, was achieved by incubating the slab gel for 16 h in denaturant containing 12% (w/v) trichloroacetic acid, 3.5% (w/v) sulphosalicylic acid and 30% (v/v) propan-2-ol prior to staining with Coomassie Blue.

Immunoblots were developed with avidin/biotin/peroxidase and quantified by soft laser densitometry with reference to internal standards, as before [13]. Antiserum to rat calbindin$_{9kDa}$ was obtained from a New Zealand White rabbit hyperimmunized by multiple subcutaneous (calbindin$_{9kDa}$ emulsified in Freund's adjuvant) and intraperitoneal (calbindin$_{9kDa}$ immobilized on finely diced polyvinylidene fluoride membrane) injections, as described for sheep [14]. Trials with dot-blots and immunoblots (results not shown) revealed that the antiserum was monospecific for calbindin$_{9kDa}$ at a dilution of 1:500, that immunoreactivity was independent of calcium (0–5 mM), and that the detection limit was equivalent for native and denatured antigen. Calcium-binding proteins were detected by ^{45}Ca overlay [15]. The calcium-binding properties of calbindin$_{9kDa}$ prepared with and without perchloric acid were compared at several loadings (0.5–2.0 μg) in the linear range, using autoradiographs quantified by laser densitometry as above. The overlay solution contained 5 μM $^{45}CaCl_2$ in 25 mM Tris/HCl, pH 7.5, 100 mM KCl and 1.5 mM $MgCl_2$. In some of the perchloric acid solubility experiments, brain cytosol was supplemented with purified protein S100A or S100B to enhance detection.

Other procedures

The protein concentration of cytosol was determined by a dye-binding assay [16], with BSA (fraction V; USB) as the standard ($A^{1\%}_{1cm,280}$ 6.5).

To prepare the fragment comprising residues 55–74 (tryptic peptide 8 [17]), calbindin$_{9kDa}$ (purified by perchloric acid precipitation and r.p.-h.p.l.c. as above) was incubated with trypsin [enzyme/substrate = 1:20 (w/w)] in a solution containing 100 mM Tris/HCl, pH 8.0, 10% (v/v) acetonitrile and 0.02% (v/v) Thesit (a non-ionic detergent with low u.v. absorbance; Boehringer Mannheim) for 16 h at 37 °C. Calbindin$_{9kDa}$ was totally fragmented under these conditions. The digest was made 1% (v/v) in trifluoroacetic acid and subjected to r.p.-h.p.l.c. on a 2.1 mm × 100 mm octadecyl column (Aquapore; ABI) as described for calbindin$_{9kDa}$ (see above and Figure 3 legend), except that the elution gradient commenced at 0% acetonitrile. The desired tyrosine-containing peptide was identified by on-line spectral analysis and with reference to the tryptic peptide map in [17]. Peptide samples (~ 90 pmol) were immobilized on Polybrene-coated glass fibre discs, and automated N-terminal sequencing was carried out with a gas phase instrument (model 470A-R/120A/610A; ABI) using the enhanced operating conditions described in [18]. The N-termini of calbindin$_{9kDa}$ samples (~ 200 pmol) were analysed in the same way.

RESULTS

Calbindin$_{9kDa}$ is soluble in 7% (v/v) perchloric acid

A dense flocculate formed immediately on addition of one-tenth volume of perchloric acid to rat duodenum cytosol. Analysis of the supernatant by SDS/PAGE revealed that nearly all (> 95%) proteins were precipitated. Of the few proteins remaining soluble in perchloric acid, two major bands exhibited the same mobilities as calbindin$_{9kDa}$ and ubiquitin (Figure 1, lanes 1–3). In comparison, precipitation at 100 °C removed fewer proteins than did perchloric acid. Most heat-stable proteins, except calbindin$_{9kDa}$ and ubiquitin, were removed by subsequent exposure to perchloric acid (Figure 1, lanes 4 and 5).

Immunoblot analyses showed that calbindin$_{9kDa}$ remained immunoreactive after exposure to perchloric acid. It was clear that most (≥ 75%) cytosolic calbindin$_{9kDa}$ was recovered in the perchloric acid-soluble fraction (Figure 2, lanes 1–3). Attempts to quantify this proportion more accurately by densitometry were thwarted by the consistent finding of less immunoperoxidase

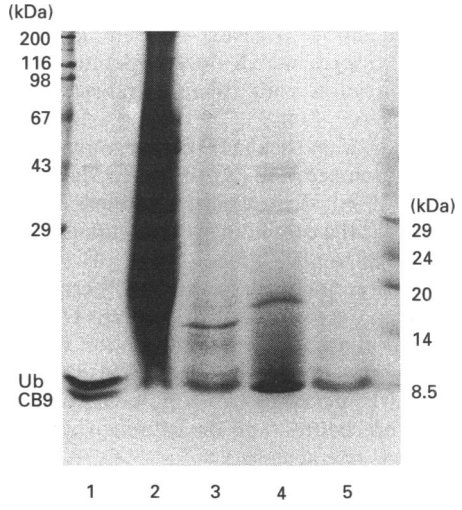

Figure 1 SDS/PAGE analysis of perchloric acid- and heat-stable proteins in rat duodenum cytosol

Cytosol was treated with 7% (v/v) perchloric acid or was heated at 100 °C, and then the soluble fractions were electrophoresed on a 10–20% linear gradient SDS/polyacrylamide gel and stained with Coomassie Blue. Molecular mass (kDa) markers are indicated at the sides. Lane 1, 0.7 μg of rat calbindin$_{9kDa}$ (CB9) and 1 μg of ubiquitin (Ub) standards; lane 2, 4 μl of cytosol; lane 3, 4 μl of perchloric acid supernatant; lane 4, 4 μl of 100 °C-treated supernatant; lane 5, 4 μl of supernatant obtained after treatment with both 100 °C and perchloric acid.

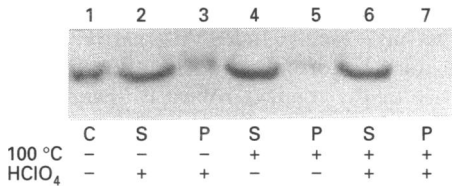

Figure 2 Immunoblot analysis of calbindin$_{9kDa}$ solubility during perchloric acid and heat treatment

Cytosol (C) was treated by heating (100 °C) and/or with perchloric acid ($HClO_4$) as indicated, centrifuged, and the supernatants (S) collected. The pellets (P) were resuspended to the original volume by sonication in SDS/PAGE sample-loading buffer and neutralized. Samples (1 μl) were electrophoresed as in Figure 1 and the immunoblot was developed with anti-calbindin$_{9kDa}$. No immunostaining was detected outside the 9 kDa region illustrated. This experiment was repeated four times with the same result. The electrophoretic mobilities of calbindin$_{9kDa}$ differed in S and P fractions due to differences in the amount of sample loaded (cf. Figure 1).

reaction product in the cytosol (lane 1) than in the subsequent supernatant and pellet fractions. A similarly high proportion of calbindin$_{9kDa}$ remained soluble after heating to 100 °C (Figure 2, lanes 4 and 5). Strikingly, little or no calbindin$_{9kDa}$ was detected in the pellet obtained after exposure of the heat-stable fraction to perchloric acid (lanes 6 and 7), clearly revealing its near-quantitative solubility.

Rapid purification of calbindin$_{9kDa}$

A relatively simple chromatogram was obtained on r.p.-h.p.l.c. of the perchloric acid-soluble fraction of rat duodenum cytosol, with major peaks corresponding to calbindin$_{9kDa}$ and ubiquitin. The calbindin$_{9kDa}$ peak was fully resolved from other components (Figure 3a) and the first derivative of the absorbance at 214 nm showed simple Gaussian characteristics (Figure 3a, inset), indicating purity.

The two-step (perchloric acid treatment and r.p.-h.p.l.c.) procedure was readily scaled up to produce milligram amounts of calbindin$_{9kDa}$. Most preparations exhibited a u.v. spectrum with the characteristic 250–270 nm Phe detailing expected for a pure protein with the aromatic composition (6 Phe, 2 Tyr [17]) of rat calbindin$_{9kDa}$. When necessary, an additional anion-exchange f.p.l.c. step (see the Experimental section) was used to remove contaminants that obscured this spectral feature. Purified calbindin$_{9kDa}$ migrated as a single species, pI 4.9, during denaturing two-dimensional PAGE, and in the ^{45}Ca-overlay procedure it exhibited calcium-binding characteristics indistinguishable from those of calbindin$_{9kDa}$ not exposed to perchloric acid (results not shown). To test for proteolysis, a 4-month-old preparation of perchloric acid-treated calbindin$_{9kDa}$ was subjected to automated Edman sequence analysis. No residues were detected, consistent with complete preservation of the acetylated N-terminus [7,17].

Residue 59 (Asn) of rat calbindin$_{9kDa}$ might be susceptible to deamidation (see the Discussion section). Sequence analysis of the tryptic peptide comprising residues 55–74 gave Glu-Leu-Asp-Lys-Asn-Gly-Asp-Gly-Glu-Val-Ser-Tyr-Glu-Glu-Phe-Glu-Val-Phe-Phe-Lys, as predicted by analyses of genomic and cDNA clones [19,20]. Assessment of Asn and Asp derivatives recovered during sequence cycles 4–6 indicated that Asn^{59} was fully amidated, since the amount of Asp did not rise above background (results not shown).

Calbindin$_{9kDa}$ was purified from the duodenum of 8-week-old rats, using perchloric acid and r.p.-h.p.l.c., with a yield of 21.4±2.3 nmol/g wet weight of tissue (mean±S.E.M.; n = 3).

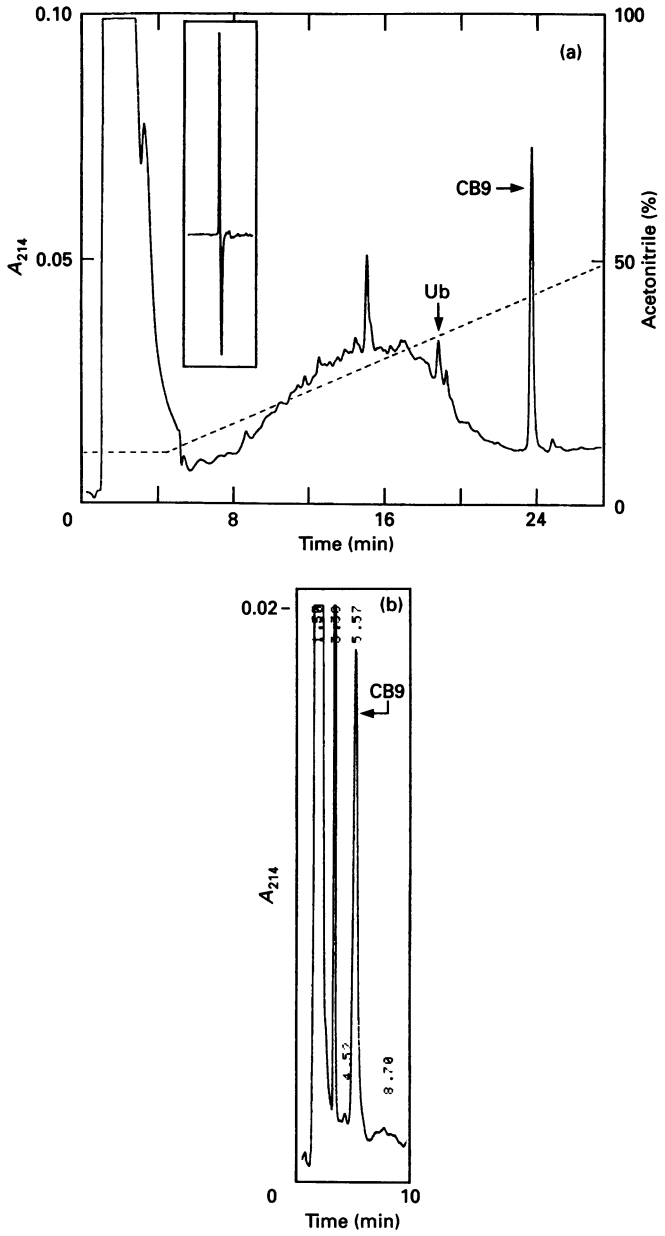

Figure 3 Reverse-phase h.p.l.c. purification of calbindin$_{9kDa}$ from the perchloric acid-soluble fraction of rat duodenum cytosol

(**a**) The supernatant of perchloric acid-treated cytosol (2.5 μl) was made 10% (v/v) in acetonitrile, applied at zero time to a 2 mm × 100 mm butyl column, and eluted with the indicated acetonitrile gradient (broken line) at a flow rate of 0.2 ml/min. Further details appear in the Experimental section. The eluate was monitored at 214 nm with a diode array detector (Model 1000S; from ABI) in zero-order (main panel) or first-derivative (inset) mode. Calbindin$_{9kDa}$ (CB9) and ubiquitin (Ub) peaks are indicated. The inset shows the first-derivative peak obtained when a sample of the calbindin$_{9kDa}$ peak (CB9) was re-chromatographed as above. (**b**) For isocratic separation, the supernatant of perchloric acid-treated rat duodenum cytosol (2 μl) was made 38% (v/v) in acetonitrile and applied at zero time to a 2.1 mm × 100 mm octyl column equilibrated with 0.1% (v/v) trifluoroacetic acid and 38% (v/v) acetonitrile flowing at 0.2 ml/min. The calbindin$_{9kDa}$ peak (CB9) eluted at 5.57 min as indicated.

When the perchloric acid step was replaced by heating to 100 °C, with and without a subsequent perchloric acid step (cf. Figure 1), the yields of calbindin$_{9kDa}$ were 21.9±2.0 and 23.2±2.0 nmol/g (n = 3) respectively. In these experiments the protein content of duodenum cytosol was 22.8±0.7 mg/g (n = 3).

A simple direct microassay for calbindin$_{9kDa}$

The high recovery of calbindin$_{9kDa}$ in the perchloric acid supernatant (Figure 2) and the well-resolved calbindin$_{9kDa}$ peak during r.p.-h.p.l.c. (Figure 3a) prompted the development of a sensitive assay utilizing narrow-bore h.p.l.c. with isocratic elution. As shown in Figure 3(b), calbindin$_{9kDa}$ was resolved as a single peak that eluted less than 6 min after injection when the column was developed isocratically with 38% (v/v) acetonitrile. In this experiment the calbindin$_{9kDa}$ (28 pmol) present in 2 μl of duodenum cytosol was readily detected (Figure 3b). Calibration experiments with a pure standard were linear ($r > 0.999$) from 100 pmol to below 1 pmol of calbindin$_{9kDa}$ (results not shown).

Other potential applications

The applicability of the perchloric acid precipitation procedure to another species was tested with the rabbit protein as in Figure 2, but with ^{45}Ca-overlay replacing immunodetection. Rabbit duodenum calbindin$_{9kDa}$ was quantitatively recovered in the perchloric acid-soluble fraction (results not shown), and subsequent r.p.-h.p.l.c. gave a yield of 65 ± 2.5 nmol/g wet weight of tissue ($n = 3$).

To test the perchloric acid solubility of other calcium-binding proteins, cytosol preparations from rat brain and skeletal muscle were made 7% (v/v) in perchloric acid or heated to 100 °C, centrifuged, and the supernatants were analysed by ^{45}Ca-overlay together with purified calcium-binding protein standards. While S100, parvalbumin, calmodulin, calbindin$_{28kDa}$ and calbindin$_{30kDa}$ (calretinin) were recovered in the heat-treated supernatants, no (functional) calcium-binding proteins were detected after precipitation with perchloric acid (results not shown).

DISCUSSION

The denaturing properties of perchloric acid are widely utilized to precipitate macromolecules. With brief exposures at low temperatures and no added calcium chelator, calbindin$_{9kDa}$ appeared to be almost totally soluble in 7% (v/v) perchloric acid. While several proteins at least partially resisted precipitation with perchloric acid or with heat, only calbindin$_{9kDa}$ and one other major protein were detected after treatment with both denaturants (Figure 1), emphasizing the rareness of such stability. The second ~ 9 kDa protein was identified as ubiquitin by N-terminal sequencing (M. J. Hubbard, unpublished work), confirming the solubility at 90 °C and in perchloric acid previously reported for yeast ubiquitin [21]. The resistance of calbindin$_{9kDa}$ and ubiquitin to denaturation might arise from the compact tertiary structures [8,22] shared by these small proteins.

Many EF-hand calcium-binding proteins are relatively stable, particularly to heating and proteolysis. This study showed that, at least for those heat-stable EF-hand proteins tested (S100, parvalbumin, calmodulin, calbindin$_{28kDa}$, calbindin$_{30kDa}$), solubility in perchloric acid is not a general feature. Calgranulins A and B were recently purified from the perchloric acid-soluble fraction of human sputum [23]. While the extent of solubility was not reported, it is interesting that these small (10.8 kDa and 13.2 kDa) EF-hand proteins belong to the same family (S100) as calbindin$_{9kDa}$.

The remarkable stability of calbindin$_{9kDa}$ might provide a clue to its unknown function(s). For instance, the similarly rugged characteristics exhibited by ubiquitin are complementary to its known involvement in protein degradation, heat shock response and cell surface events [22]. While current understanding of calbindin$_{9kDa}$ does not point to an involvement in these or similar functions, neither has it been excluded. Alternatively, the stability of calbindin$_{9kDa}$ may be fortuitous, arising from the minimalist expression of two tightly coupled, robust EF-hand domains.

The apparent purity of calbindin$_{9kDa}$ following r.p.-h.p.l.c. of perchloric acid-treated rat duodenum cytosol (Figure 3a) was confirmed by denaturing two-dimensional PAGE, u.v. spectral analysis and N-terminal sequencing. Compared with published procedures (e.g. [11,17,24]), this two-step purification procedure has the clear benefits of simplicity (technique and equipment), speed (readily completed in 1 day), avoidance of proteolysis and high recovery. The amount of calbindin$_{9kDa}$ in our duodenum preparations from normally fed juvenile rats, i.e. approx. 30 nmol/g wet weight of tissue or 12 μg/mg of soluble protein (estimated from the immunoblotting and ^{45}Ca-overlay data, and comparable with published values [25,26]), indicates that the reported yields (see the Results section) represented a recovery of approx. 70%.

The possibility of introducing artefactual modifications is of major concern when using denaturing steps during protein purification. Indeed, some purification protocols have resulted in partial deamidation of calbindin$_{9kDa}$ [27,28]. Rat calbindin$_{9kDa}$ purified with the perchloric acid procedure exhibited no heterogeneity during isoelectric focusing and anion-exchange f.p.l.c., indicating no such alteration in charge. The ^{45}Ca-overlay data implied that the calcium-binding properties of calbindin$_{9kDa}$ were unaffected by exposure to perchloric acid. Likewise, in preliminary equilibrium binding experiments using a (native) dot-blot assay essentially as before [13], the apparent calcium-binding constants were the same for calbindin$_{9kDa}$ purified with and without perchloric acid (M. J. Hubbard, unpublished work). These findings notwithstanding, the potential for perchloric acid to modify the functional properties of calbindin$_{9kDa}$ should be recognized by investigators using this purification procedure.

Genomic and cDNA cloning analyses of rat calbindin$_{9kDa}$ predicted the presence of Asn at residue 59 [19,20], whereas sequencing of the purified intestinal and placental proteins gave Asp at this position [17]. Since the other Asn residues were correctly identified [17], this discrepancy might reflect a particular susceptibility of Asn^{59} to deamidation during the protein purification or analytical procedures, as noted previously [19,20]. The present study first established that residue 59 in the rat protein is indeed Asn, and secondly showed that the perchloric acid-based purification procedure did not cause deamidation of this amino acid.

Two isoforms of calbindin$_{9kDa}$ were detected in preparations from the duodenum of pig [29] and mouse, where they were resolved by r.p.-h.p.l.c. and SDS/PAGE [28]. The finding in the present study that perchloric acid-treated calbindin$_{9kDa}$ was homogeneous by r.p.-h.p.l.c. and two-dimensional PAGE makes it unlikely that different isoforms exist in the duodenum of the Wistar rat. Calbindin$_{9kDa}$ purified from rabbit duodenum also appeared as a single peak on r.p.-h.p.l.c.

Use of the two-step procedure as a microassay gave the advantage of direct quantification of calbindin$_{9kDa}$ by measuring the peptide bond absorbance at 214 nm. The ability to resolve calbindin$_{9kDa}$ under isocratic conditions would enable this assay to be carried out rapidly with a single-pump h.p.l.c. The sensitivity of this microassay, below 1 pmol with narrow-bore r.p.-h.p.l.c., was higher than that of our avidin/biotin-amplified immunoblot assay. In contrast, radioimmunoassay was useful down to 0.1 pmol of calbindin$_{9kDa}$ [25] but, in addition to being indirect and possibly non-specific, this and other immunodetection procedures are limited by the widely reported species-specificity of anti-calbindin$_{9kDa}$ [1].

The perchloric acid-based micropurification will be of particular use for investigating calbindin$_{9kDa}$ in previously intractable low-abundance tissue sources, such as cultured cells. In a preliminary investigation of samples (400 μg of cytosolic protein) from 8-week-old rat kidney, an r.p.-h.p.l.c. peak with the retention time of calbindin$_{9kDa}$ was barely discerned, suggesting that the renal antigen detected by immunohistochemistry [1,30] represents a minor component of the mature organ, as concluded by others [1]. In contrast, calbindin$_{9kDa}$ was clearly identified in rat dental enamel epithelium (a diminutive tissue isolated from developing teeth), where it appeared to account for only $\sim 0.02\%$ of the soluble proteins (M. J. Hubbard, unpublished work).

In conclusion, this study has established a new property of calbindin$_{9kDa}$, i.e. solubility in perchloric acid, which provides a powerful approach to purification and microassay. It is envisaged that these procedures will find particular utility for investigations of calbindin$_{9kDa}$ in previously intractable low-abundance sources.

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REFERENCES

1 Christakos, S., Gabrielides, C. and Rhoten, W. B. (1989) Endocr. Rev. **10**, 3–26
2 Gross, M. and Kumar, R. (1990) Am. J. Physiol. **259**, F195–F209
3 Mathieu, C. L., Mills, S. E., Burnett, S. H., Cloney, D. L., Bruns, D. E. and Bruns, M. E. (1989) Endocrinology (Baltimore) **124**, 2745–2750
4 Li, H. and Christakos, S. (1991) Endocrinology (Baltimore) **128**, 2844–2852
5 Dalgarno, D. C., Levine, B. A., Williams, R. J. P., Fullmer, C. S. and Wasserman, R. H. (1983) Eur. J. Biochem. **137**, 523–529
6 Wendt, B., Hofmann, T., Martin, S. R., Bayley, P., Brodin, P., Grundstrom, T., Thulin, E., Linse, S. and Forsen, S. (1988) Eur. J. Biochem. **175**, 439–445
7 Fullmer, C. S. and Wasserman, R. H. (1981) J. Biol. Chem. **256**, 5669–5674
8 Akke, M., Drakenburg, T. and Chazin, W. J. (1992) Biochemistry **31**, 1011–1020
9 Mach, H., Middaugh, C. R. and Lewis, R. V. (1992) Anal. Biochem. **200**, 74–80
10 Schagger, H. and Von Jagow, G. (1987) Anal. Biochem. **166**, 368–379
11 Bruns, M. E. H., Fausto, A. and Alvioli, L. V. (1978) J. Biol. Chem. **253**, 3186–3190
12 Hochstrasser, D. F., Harrington, M. G., Hochstrasser, A. C., Miller, M. J. and Merril, C. R. (1988) Anal. Biochem. **173**, 424–435
13 Hubbard, M. J. and Klee, C. B. (1989) Eur. J. Biochem. **185**, 411–418
14 Hubbard, M. J. and Cohen, P. (1991) Methods Enzymol. **201**, 414–427
15 Maruyama, K., Mikawa, T. and Ebashi, S. (1984) J. Biochem. (Tokyo) **95**, 511–519
16 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
17 MacManus, J. P., Watson, D. C. and Yaguchi, M. (1986) Biochem. J. **235**, 585–595
18 Tempst, P. and Riviere, L. (1989) Anal. Biochem. **183**, 290–300
19 Darwish, H. M., Krisinger, J., Strom, M. and DeLuca, H. F. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 6108–6111
20 Perret, C., Lomri, N., Gouhier, N., Auffray, C. and Thomasset, M. (1988) Eur. J. Biochem. **172**, 43–51
21 Wilkinson, K. D., Cox, M. J., O'Connor, L. B. and Shapira, R. (1986) Biochemistry **25**, 4999–5004
22 Finley, D. (1991) Annu. Rev. Cell Biol. **7**, 25–69
23 Longbottom, D., Sallenave, J. M. and van Heyningen, V. (1992) Biochim. Biophys. Acta **1120**, 215–222
24 Gleason, W. A. and Lankford, G. L. (1981) Anal. Biochem. **116**, 256–263
25 Thomasset, M., Parkes, C. O. and Cuisinier-Gleizes, P. (1982) Am. J. Physiol. **243**, E483–E488
26 Roche, C., Bellaton, C., Pansu, D., Miller, A. and Bronner, F. (1986) Am. J. Physiol. **251**, G314–G320
27 Chazin, W. J., Kordel, J., Thulin, E., Hofmann, T., Drakenburg, T. and Forsen, S. (1989) Biochemistry **28**, 8646–8653
28 Hunt, D. F., Yates, J. R., Shabanowitz, J., Bruns, M. E. and Bruns, D. E. (1989) J. Biol. Chem. **264**, 6580–6586
29 Hofmann, T., Kawakami, M., Hitchman, A. J. W., Harrison, J. E. and Dorrington, K. J. (1979) Can. J. Biochem. **57**, 737–748
30 Bindels, R. J. M., Timmermans, J. A. H., Hartog, A., van Os, C. H. and Coers, W. (1991) J. Am. Soc. Nephrol. **2**, 1122–1129

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