Biochimica et Biophysica Acta 896 (1987) 275–286
Elsevier

BBA 73348

The mode of anchoring and precursor forms of sucrase-isomaltase and maltase-glucoamylase in chicken intestinal brush-border membrane. Phylogenetic implications

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(Received 17 July 1986)

Key words: Sucrase-isomaltase; Maltase-glucoamylase; Membrane anchoring; Phylogeny; (Chicken intestine)

Chicken intestinal sucrase-isomaltase and maltase-glucoamylase have been isolated in their intact form by detergent solubilization and characterized as to their subunit composition and mode of anchoring in the brush-border membrane. Both are heterodimeric enzyme complexes composed of two subunits each of approximately 140 and 130 kDa. Contrary to the mammalian sucrase-isomaltase, chicken isomaltase was identified as the smaller of the two subunits. As was shown by hydrophobic labeling, only one of the two subunits in each heterodimer is anchored in the bilayer, the smaller 130 kDa isomaltase subunit of the sucrase-isomaltase complex, and the larger 140 kDa subunit of the maltase-glucoamylase complex. Both preparations contain a high-molecular weight polypeptide of approximately 250 kDa which in the case of sucrase-isomaltase could be identified by peptide mapping as a single-chain precursor not (yet) proteolytically processed to the final heterodimer. These first data on the mode of membrane anchoring of non-mammalian glycosidases indicate that they are synthesized, inserted into the membrane, and processed in ways similar to the mammalian enzymes. The fundamental unity between avian and mammalian sucrase-isomaltases suggests that the partial gene duplication of an ancestral isomaltase gene and the subsequent mutation of one of the active sites resulting in pro-sucrase-isomaltase has occurred prior to the separation of mammals from reptiles, i.e. more than 300 million years ago.

Introduction

The heterodimeric glycosidases of the mammalian small-intestinal brush border (sucrase-isomaltase; maltase-glucoamylase and the lactase-glycosylceramidase complex) are a remarkably homogeneous group of enzymes in their catalytic properties, their mode of insertion in the membrane and their biosynthesis. Each of them is synthesized as a single very long polypeptide (larger than 220 kDa), which is split posttranslationally into two similar, but not identical subunits (for reviews, see Refs. 1–3). Most information stems from work on the sucrase-isomaltase complex, for which the original 'one-chain, two-active sites precursor' hypothesis encompassing both ontogeneic and phylogenetic aspects was formulated (see Discussion).

No information had been available thus far on the mode of membrane anchoring and, by inference, the biosynthesis of non-mammalian sucrase-isomaltase and maltase-glucoamylase, al-

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Abbreviation: [^{125}I]TID, 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine.

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though the former had been isolated (by proteolytic solubilization) from chicken [4–6] and from Beijing duck [7], and both (by proteolytic and detergent solubilization) from pigeon [8].

This kind of information is, however, necessary to better understand the phylogeny of these enzymes. We have thus isolated (detergent solubilized) chicken sucrase-isomaltase (sucrose α-D-glucohydrolase, EC 3.2.1.48, and isomaltase, EC 3.2.1.10) and investigated its subunit structure, its mode of anchoring to the membrane, and the existence of a large single-polypeptide chain precursor (pro-SI). In addition we have carried out similar experiments for chicken maltase-glucoamylase (γ-amylase, α-1,4-glucan glucohydrolase, EC 3.2.1.3).

Materials and Methods

General methods

Protein was determined by the Lowry procedure as modified by Peterson [9]. Sucrase, isomaltase, palatinase, maltase, and glucoamylase activities were determined by measuring the amount of glucose liberated from sucrose, isomaltose, palatinose (33 mM each), maltose (20 mM) and amylose (Sigma, type III, from potato; 0.8%, w/v), respectively, at 37°C in 33 mM sodium maleate buffer (pH 6.2). D-Glucose was determined using the glucose dehydrogenase kit from Merck.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the discontinuous system described by Brunner et al. [10] (Tris-HCl), unless stated otherwise. Protein samples were denatured in 2% SDS, 5% mercaptoethanol for 2 min at 100°C. The slab gels were stained in 0.05% Coomassie brilliant blue R-250 or with silver according to Oakley et al. [11]. For autoradiography, dried gels were exposed to Kodak X-Omat S films at −80°C.

Isolation of sucrase-isomaltase and maltase-glucoamylase

Chicken small-intestinal brush-border vesicles were prepared from frozen small intestines according to the procedure of Kessler et al. [12]. These membranes were extracted with Triton X-100 (Serva) to solubilize the intact, amphipathic brush-border hydrolases. The conditions used were identical to those described earlier for rabbit sucrase-isomaltase [13]. After removal of the residual vesicles by centrifugation at 100000 × *g* for 1 h, the extract was fractionated by gel filtration on Sephadex G-200 (Pharmacia) followed by ion exchange chromatography on DEAE-Sephadex A-25 [5]. All buffers contained 0.5% (w/v) Triton X-100. On Sephadex G-200 sucrase-isomaltase is eluted in the void volume, while maltase-glucoamylase is retarded and elutes over two to three column volumes. This retardation is due to affinity of this enzyme to the column material. Elution of maltase-glucoamylase in a small volume can be induced by increasing the temperature to 37°C or by adding maltose (100 mM) to the elution buffer. The void volume fractions were adsorbed to DEAE-Sephadex A-25 in 10 mM sodium phosphate (pH 6.8), and sucrase-isomaltase was eluted by a linear NaCl gradient (0–300 mM).

To concentrate dilute solutions of sucrase-isomaltase or maltase-glucoamylase, the enzymes were dialyzed against 10 mM sodium phosphate (pH 6.8), adsorbed to Tris-Sepharose 6B (prepared from Epoxy-activated Sepharose 6B as described by Reiss and Sacktor; [14]), and eluted in a small volume by increasing the ionic strength with 100 mM sodium phosphate. (The fact that they could not be eluted with 0.5 M glucose suggests that Tris-Sepharose acts as an ion exchanger rather than an affinity matrix as had been suggested [14].) Triton X-100 was removed by washing and eluting the enzymes after adsorption to Tris-Sepharose 6B with detergent-free buffers or, alternatively, by the procedure of Holloway [15] using Bio-Beads SM-2 (Bio-Rad). The enzymes did not precipitate, a common observation with proteins in which the hydrophobic portion does not exceed a few percent; also, it is quite possible that the detergent may not have been totally removed.

Separation of the sucrase and isomaltase subunits

Dissociation of the sucrase-isomaltase complex into enzymatically (partially) active subunits was accomplished by citraconylation as described previously for the rabbit complex [10]. To a sucrase-isomaltase solution of approx. 5 mg in 1 ml of 50 mM sodium phosphate (pH 8.2), 0.5% Triton X-100, a total of 60 μl of 10% (w/v) citraconic anhydride (Fluka) dissolved in dioxane

was added within 1 h at room temperature while the pH was kept at 8.2 by addition of 1 M NaOH. Following extensive dialysis against 10 mM sodium phosphate (pH 8.2), 0.5% Triton X-100, the citraconylated sucrase and isomaltase, whose specific enzymatic activities at this point were reduced to 5% and 15%, respectively, of their original activities, were adsorbed to a 0.5 ml-column of Tris-Sepharose 6B equilibrated in the same buffer. On washing the column with a linear gradient of 0–200 mM NaCl in the same buffer, sucrase was eluted between 50 and 120 mM NaCl and isomaltase between 140 and 190 mM NaCl, as shown in Fig. 4. Decitraconylation by dialysis against 20 mM sodium acetate (pH 5.0) for two days at room temperature, restored most of the enzymatic activities, allowing an unambiguous identification of the subunits.

Hydrophobic labeling with [^{125}I]TID

The hydrophobic, photoactivatable reagent 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) was prepared according to Ref. 14 at a specific radioactivity of 10 Ci/mmol (Na^{125}I was from EIR, Würenlingen, Switzerland). Labeling of chicken brush-border membrane vesicles was performed as previously described for rabbit membranes [16]. The labeled vesicles were washed four times with 50 mM sodium phosphate buffer (pH 7.5), containing 1% (w/v) bovine serum albumin and twice with albumin-free buffer.

Sucrase-isomaltase egg phosphatidylcholine proteoliposomes were reconstituted from isolated sucrase-isomaltase and egg phosphatidylcholine (Lipid Products, South Nutfield, Surrey, U.K.) at a lipid to protein ratio of 5 : 1 by the cholate-removal method [17] and labeled as described before [16]. The labeled proteoliposomes were solubilized with 2% sodium cholate (Merck) and the protein was separated from labeled lipid and other photolysis products of [^{125}I]TID by gel filtration on Bio-Gel A-5m (Bio-Rad) in 2% sodium cholate/50 mM sodium phosphate (pH 7.5).

Peptide mapping

Sucrase, isomaltase, and the 250 kDa protein were first separated by SDS-gel electrophoresis on a 6% polyacrylamide gel using the borate-sulfate buffer system described by Neville [18], which gave a somewhat better separation of the subunits than other systems tested. Regions containing the individual polypeptides were cut out of the gel and either cleaved with *N*-chlorosuccinimide (Sigma) by the protocol of Lischwe and Ochs [19] or by limited digestion with *Staphylococcus aureus* V8 protease (Miles) according to the procedure of Cleveland et al. [20] modified as follows: Gel slices containing the individual polypeptides (approx. 5–20 μg) were incubated for 1 h in water and for 1 h in stacking gel buffer containing 0.1% SDS. They were then fitted into the sample wells of a second SDS-gel (13% acrylamide) and covered with 50 μl of stacking gel buffer containing 0.1% SDS, 20% glycerol and 0–5 μg of V8 protease. The gel was run overnight at room temperature with 1.75 mA/cm^2.

Results

Isolation and enzymatic properties of chicken sucrase-isomaltase and maltase-glucoamylase

In the few cases investigated, sucrase-isomaltase (SI) from mammals (rabbit, rat, hog and man) are often immunologically crossreactive (although not very strongly, e.g. Refs. 21,22). In contrast, chicken sucrase-isomaltase was not recognized by anti-rabbit SI antisera raised in goat and guinea pig and by anti-hog SI antiserum raised in rabbits (as judged by immunodiffusion, Western blotting, or precipitation tests; data not shown). Therefore, to isolate the intact chicken sucrase-isomaltase complex, we used a procedure based on protocols described before for the purification of the papain-solubilized chicken enzymes [5,6]. In short, a Triton extract of isolated small-intestinal brush-border membranes was fractionated by gel filtration on Sephadex G-200 and by ion exchange chromatography (see Materials and Methods). The purification is summarized in Table I.

Gel filtration on Sephadex G-200 is essential to separate the sucrase-isomaltase (SI) and the maltase-glucoamylase (MG) complexes. Chicken maltase-glucoamylase, but not sucrase-isomaltase, is strongly retarded on Sephadex G-200. (This has also been observed for the rat enzymes [23,24], while the opposite is the case for the human [25,26] and rabbit [27] enzymes.) The final

TABLE I

PURIFICATION OF SUCRASE-ISOMALTASE (SI) AND MALTASE-GLUCOAMYLASE (MG)

Average numbers of at least two preparations are shown.

Fraction	Sucrase		Palatinase [a]		Maltase		Glucoamylase	Protein (%)
	spec. act. (U/mg)	recovery (%)	spec. act. (U/mg)	recovery (%)	spec. act. (U/mg)	recovery (%)	spec. act. (U/mg)	
Brush-border membrane vesicles	0.60	100	0.08	100	7.2	100		100
Triton extract	1.6	60	0.16	61	9.1	61		40
After Sephadex G-200 not retarded	2.1	35	0.24	37	8.7	23		23
after DEAE-Sephadex (final SI-preparation)	3.5	13	0.44	14	13.9	8	< 0.2	3
After Sephadex G-200 retarded (final MG-preparation)	< 0.1		< 0.1		10.8		0.77	0.5–1
Papain-solubilized sucrase-isomaltase	3.9		0.54		13.2			5

[a] Palatinose was used as an alternative substrate to assay isomaltase.

sucrase-isomaltase and maltase-glucoamylase preparations were essentially free of crosscontamination, as judged by measuring the hydrolase activities which the two glycosidase complexes do not have in common (glucoamylase, or sucrase and palatinase activities, respectively; Table I). Both complexes have considerable maltase activity which can be differentiated on the basis of their sensitivity to heat inactivation, maltase-glucoamylase being less stable at higher temperatures than sucrase-isomaltase (Fig. 1). The monophasic kinetics of heat inactivation of maltase activity in the final maltase-glucoamylase and sucrase-isomaltase preparations (with halftimes of inactivation $t_{1/2}$ at 61°C of approx. 3 min and 21 min, respectively) confirms their homogeneity. The close similarity of the inactivation kinetics of the maltase activity of sucrase-isomaltase with that of sucrase activity ($t_{1/2}$ at 61°C for sucrase: approx. 18 min; for palatinase: approx. 40 min) indicates that the sucrase subunit contributes most of the maltase activity of the sucrase-isomaltase complex. This is confirmed by substrate inhibition experiments which clearly showed mutual inhibition between

TABLE II

MUTUAL EFFECT OF SUCROSE, ISOMALTOSE, AND MALTOSE ON THEIR HYDROLYSIS BY SUCRASE-ISOMALTASE

The units (U) are defined as μmoles of glucose liberated at 37°C per min in 33 mM sodium maleate buffer (pH 6.2). The substrate concentrations were 33 mM, 33 mM, and 20 mM for sucrose, isomaltose, and maltose, respectively. The average values of triplicate determinations (± S.D.) are shown.

Substrate	Enzyme activity (U/ml)		Inhibition (%)
	experimental	calculated from individual substrates	
Sucrose	0.277 ± 0.009		
Isomaltose	0.600 ± 0.009		
Maltose	0.514 ± 0.002		
Sucrose + isomaltose	0.809 ± 0.019	0.877 ± 0.018	8 ± 4
Sucrose + maltose	0.530 ± 0.004	0.791 ± 0.011	33 ± 2
Isomaltose + maltose	1.002 ± 0.033	1.114 ± 0.011	10 ± 4

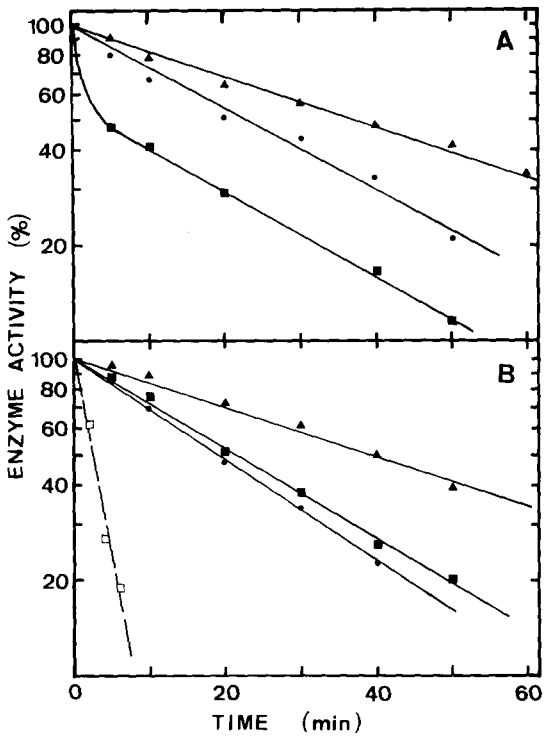


Fig. 1. Thermal inactivation of sucrase, palatinase, and maltase. Brush border membranes (panel A), purified sucrase-isomaltase (panel B, filled symbols) and purified maltase-glucoamylase (open squares) were incubated at 61°C in 10 mM sodium phosphate (pH 7.5) for different times. After cooling on ice, the remaining enzyme activities were determined. Maltase (□ and ■), sucrase (●), palatinase (▲) (palatinose was used as an alternative substrate for isomaltase).

sucrose and maltose, but only very little inhibition between isomaltose and maltose and between isomaltose and sucrose (Table II).

As judged by SDS-PAGE (Fig. 2, lane B, and Fig. 3, lane B), the final chicken sucrase-isomaltase preparation consists of two major bands of apparent molecular weights of 140 and 130 kDa, corresponding to the mature subunits of the complex, and a minor band of approx. 250 kDa, which was shown to be pro-SI, the precursor form of sucrase-isomaltase not (yet) cleaved into the subunits (see below). In addition, there are two faint bands in the 170 kDa region and another one of approx. 110 kDa which are probably contaminants. Together they account for not more than 15% of the protein.

The maltase-glucoamylase preparation is free of contaminants, since its purification is based on affinity to Sephadex G-200. The complex consists of two subunits which are nearly identical in size to those of sucrase-isomaltase (130 and 140 kDa) and also of a 250 kDa band which in all likelihood corresponds to its precursor, pro-MG (Fig. 2, lane C).

Identification and isolation of the sucrase and isomaltase subunits

In all mammalian sucrase-isomaltases studied so far, the isomaltase subunit was found to have the higher apparent molecular weight than the sucrase subunit on SDS-PAGE. In contrast, chicken isomaltase activity is associated with the 130 kDa polypeptide, i.e. the subunit with the lower apparent molecular weight, and sucrase activity with the 140 kDa subunit. This was shown by analyzing the enzyme activities and the SDS-gel mobilities of the individual subunits. To this end the sucrase-isomaltase complex was dissociated by citraconylation and fractionated by ion exchange chromatography (Fig. 4). Upon decitraconylation the isolated subunits regained a large fraction of their enzymatic activities.

The mode of anchoring of chicken sucrase-isomaltase in the membrane

Mammalian sucrase-isomaltases (exemplified by the rabbit and hog enzymes) are anchored in the brush-border membrane solely by a highly hydrophobic segment close to the amino terminus of the isomaltase subunit. This mode of anchoring was established by various criteria including the observation that the isomaltase subunit was selectively labeled from within the core of the bilayer by the hydrophobic photolabeling reagent 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine([^{125}I]-TID) [16,28].

Similarly, to identify the subunit(s) of chicken sucrase-isomaltase and maltase-glucoamylase that are embedded in the membrane, brush-border vesicles were labeled with [^{125}I]TID, sucrase-isomaltase and maltase-glucoamylase were isolated and fractionated by SDS-PAGE (Fig. 2). Autoradiography showed that of the chicken sucrase-isomaltase complex only the smaller 130 kDa isomaltase subunit was labeled, of the maltase-glucoamylase complex, on the other hand, only the

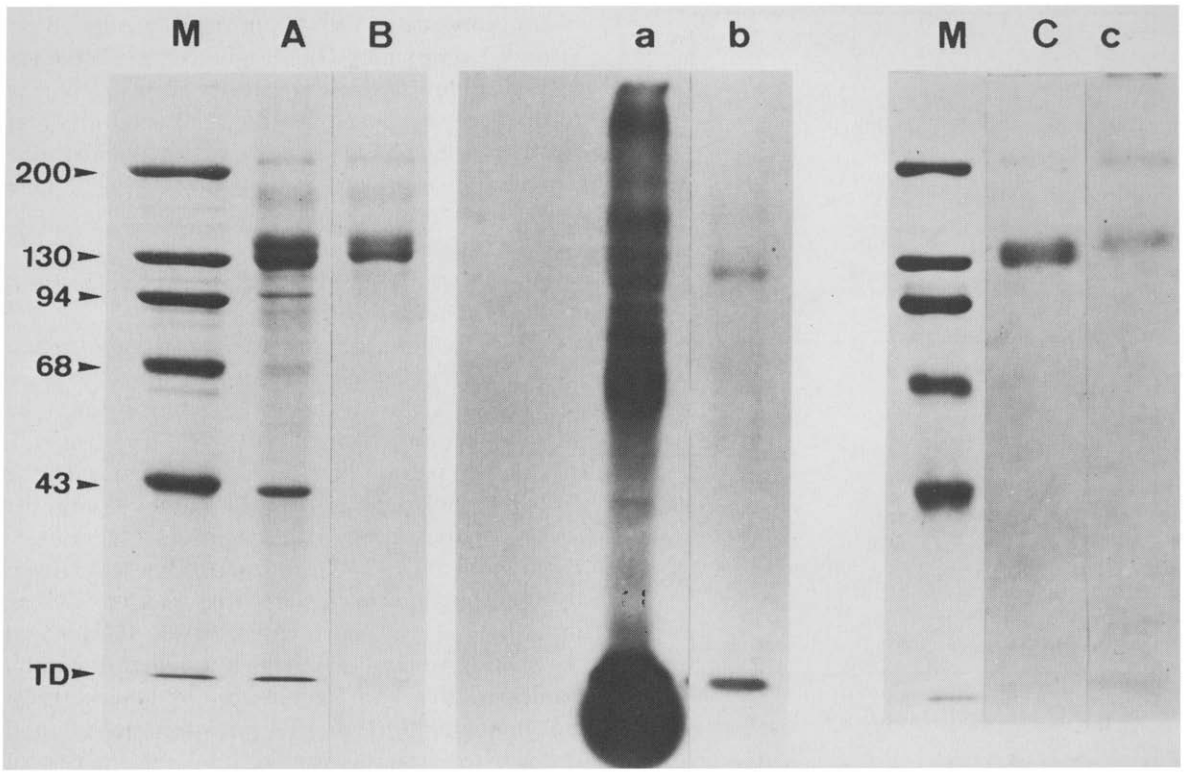
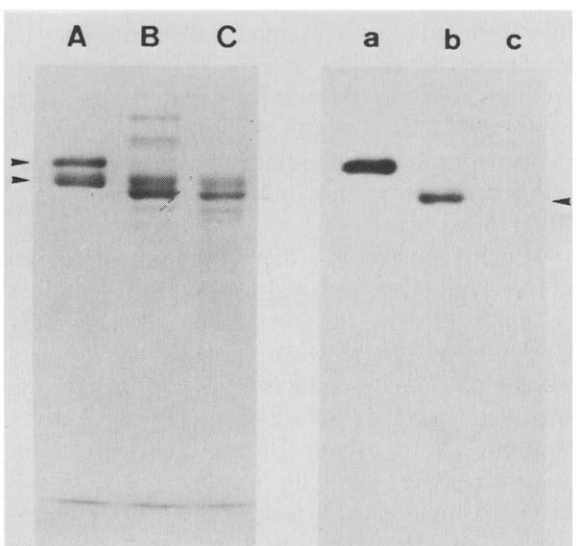


Fig. 2. Isolation and [^{125}I]TID-labeling of chicken sucrase-isomaltase and maltase-glucoamylase. Brush-border membrane vesicles labeled with [^{125}I]TID (lane A), and sucrase-isomaltase (lane B) and maltase-glucoamylase (lane C) isolated from these vesicles were analyzed by SDS-PAGE (8% acrylamide) and Coomassie blue staining. In lanes a–c the corresponding autoradiographs are shown. The marker proteins used (lane M) were myosin, β-galactosidase, phosphorylase B, bovine serum albumin, and ovalbumin. Their molecular weights are indicated in kDa.



larger 140 kDa subunit was labeled. The 250 kDa precursors of both were labeled as well. [^{125}I]TID-labeling of isolated sucrase-isomaltase after reconstitution into proteoliposomes gave the same result (data not shown).

Fig. 3. Comparison of detergent-solubilized and papain-solubilized chicken sucrase-isomaltase. [^{125}I]TID-labeled chicken sucrase-isomaltase isolated by Triton X-100 solubilization was reconstituted into sucrase-isomaltase/egg phosphatidylcholine proteoliposomes and digested with papain and purified by chromatography on Tris-Sepharose 6B. The Triton-solubilized (lane B) and papain-solubilized form (lane C) of chicken sucrase-isomaltase and, for comparison, the detergent-solubilized [^{125}I]TID-labeled rabbit sucrase-isomaltase (lane A) were analyzed by SDS-PAGE (8% acrylamide) and Coomassie staining. In lanes a–c the corresponding autoradiograph is shown. The two arrows on the left indicate the running distances of rabbit isomaltase (top, 160 kDa) and sucrase (bottom, 140 kDa); the arrow on the right indicates the running distance of chicken isomaltase (130 kDa).

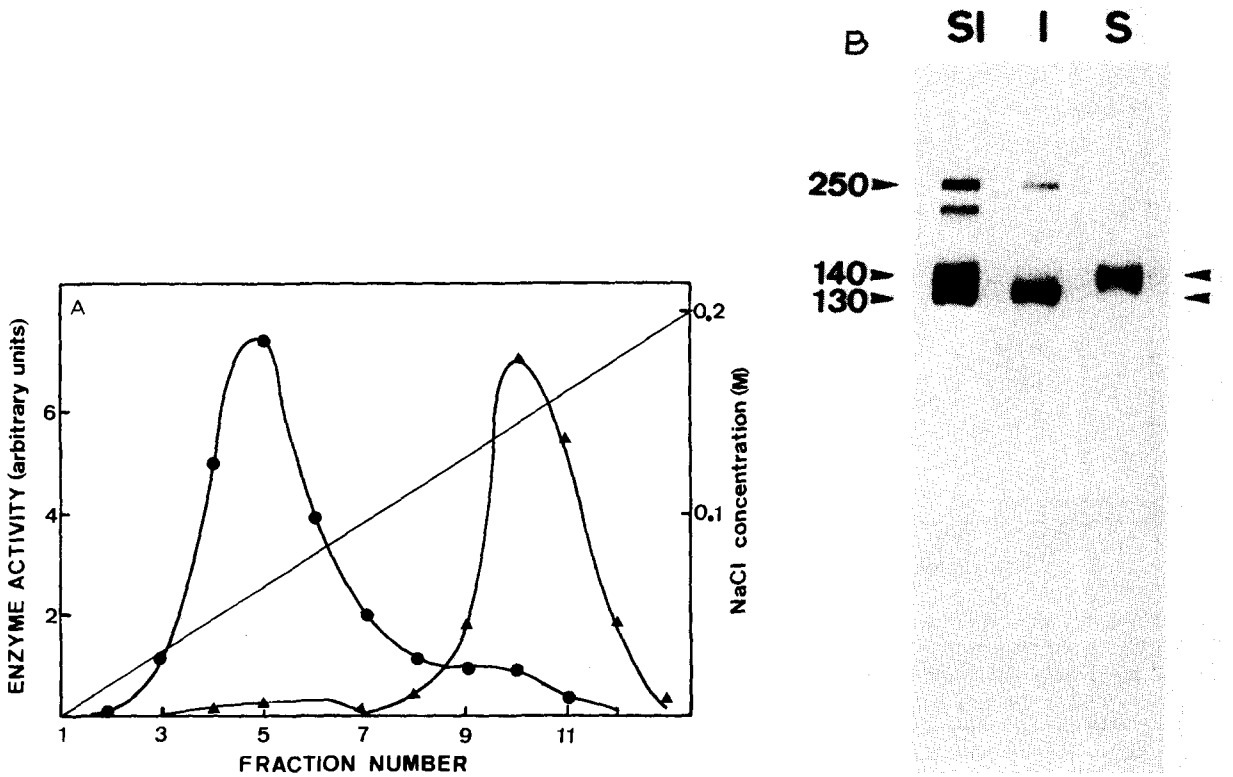


Fig. 4. Separation and identification of the subunits of the chicken sucrase-isomaltase complex (SI). Chicken sucrase-isomaltase was citraconylated and subjected to ion exchange chromatography on Tris-Sepharose 6B as described in Materials and Methods. In panel A the resulting elution profile for isomaltase (●) and sucrase (▲) activity is shown. Panel B shows the protein pattern of the isolated subunits (I and S) and of the starting material (SI) after SDS-PAGE (8% acrylamide) and silver staining.

Solubilization of sucrase-isomaltase by papain digestion produces a hydrophilic form with unaltered enzymatic properties by proteolytic removal of the segment ancoring the complex in the membrane. Accordingly, papain-solubilized sucrase-isomaltase is devoid of radioactivity, the [^{125}I] TID-labeled membranous segment of isomaltase having been cleaved off (Fig. 3, lane c). It is surprising that there is no detectable difference in the protein patterns of intact (detergent-solubilized) and papain-solubilized forms of chicken sucrase-isomaltase (Fig. 3, lanes B and C), suggesting that only a very small polypeptide segment is removed. In contrast, a change in the apparent molecular weight of approx. 20 kDa is observed for the isomaltase subunit of the rabbit complex on papain digestion [10]. It is conceivable that in rabbit sucrase-isomaltase, in addition to the anchor portion itself, also part of the 'stalk' is cleaved off, i.e. part of the segment of the polypeptide chain connecting the anchor with the globular enzyme domains.

The [^{125}I]TID-labeled membranous anchor peptide as it is produced by papain digestion of sucrase-isomaltase/egg phosphatidylcholine proteoliposomes was analyzed by SDS-PAGE and gel filtration as is shown in Fig. 5. On SDS-gel electrophoresis it runs as a well defined peptide of an apparent molecular weight of approx. 9000 Da, which is somewhat higher than the more diffuse band of approx. 8000 Da produced by the anchor segment of rabbit sucrase-isomaltase. Because of the unknown SDS binding properties of such highly hydrophobic peptides, the size determination by gelfiltration on Sephadex LH-20 in ethanol/formic acid [29] is presumably more reliable: The chicken anchor peptide elutes as a symmetric peak with an estimated molecular weight of

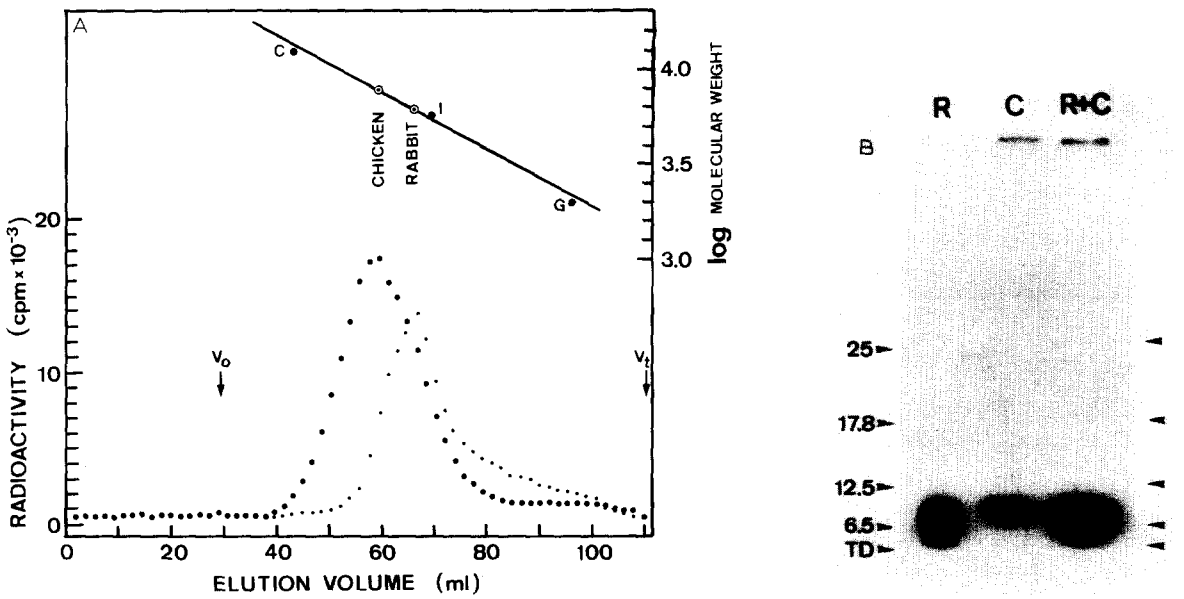


Fig. 5. Characterization of the hydrophobic anchor peptide of chicken sucrase-isomaltase. [^{125}I]TID-labeled sucrase-isomaltase from chicken or, for comparison, from rabbit was reconstituted into sucrase-isomaltase/egg phosphatidylcholine proteoliposomes and digested with papain. After adsorption of the solubilized enzyme on Tris-Sepharose 6B, the proteoliposomes containing the anchor peptides were gel filtered on a Sephadex LH-60 column (1.5×63 cm; flow rate, 7.2 ml/h) equilibrated in ethanol/formic acid (88%), 7:3 (v/v) [29], and calibrated with cytochrome *c* (C), insulin (I), and gramicidin (G). The radioactivity of both the rabbit and the chicken anchor peptides eluted as a single peak with a trailing shoulder, which is probably due to labeled lipids non-covalently associated with the peptides and which is absent in the rechromatography of the peak material. In panel A the elution profiles of the anchor peptides of rabbit sucrase-isomaltase (small dots; first chromatography) and of chicken sucrase-isomaltase (large dots; rechromatography) are shown. The [^{125}I]TID-labeled anchor peptides (R, from rabbit sucrase-isomaltase; C, from chicken sucrase-isomaltase) from the peak fractions were dried, redissolved in 2% SDS and analyzed by SDS-PAGE (15% acrylamide). 1–2 μg of the peptides did not stain with Coomassie blue. In panel B an autoradiograph is shown. The positions of the marker proteins (chymotrypsinogen A, myoglobin, cytochrome *c*, aprotinin) are indicated with the respective molecular weights in kDa.

approx. 7500 Da as compared to approx. 6500 Da for the rabbit anchor [16]. The mode of anchoring of chicken and rabbit sucrase-isomaltase appears to be essentially the same. The small differences between the two anchor peptides might merely reflect differences in the exact positions and in the homogeneity of papain cleavage in the stalk region.

Identification of the 250 kDa protein as pro-sucrase-isomaltase (pro-SI)

By analogy to the mammalian sucrase-isomaltases [30,31], it was suspected that the 250 kDa protein of the chicken sucrase-isomaltase preparation corresponds to the unprocessed precursor form of sucrase-isomaltase. This could be proven by the comparison of the protein patterns ('fingerprints') obtained by SDS-PAGE of V8 protease and *N*-chlorosuccinimide digests of the 250 kDa band and of the individual sucrase-isomaltase subunits (Fig. 6). The protein patterns obtained from sucrase and isomaltase are very similar, in agreement with the expected homology between the two subunits (Fig. 6, lanes A and B). The pattern of the 250 kDa band is essentially the superposition of those of sucrase and of isomaltase. Furthermore, autoradiography of the V8 protease digests reveals an identical pattern of [^{125}I]TID-labeled fragments for isomaltase and the 250 kDa protein. The 250 kDa band is not merely a non-dissociated heterodimer of the sucrase and isomaltase subunits, because the samples were boiled and electrophorized under dissociating conditions (see under Methods), and because

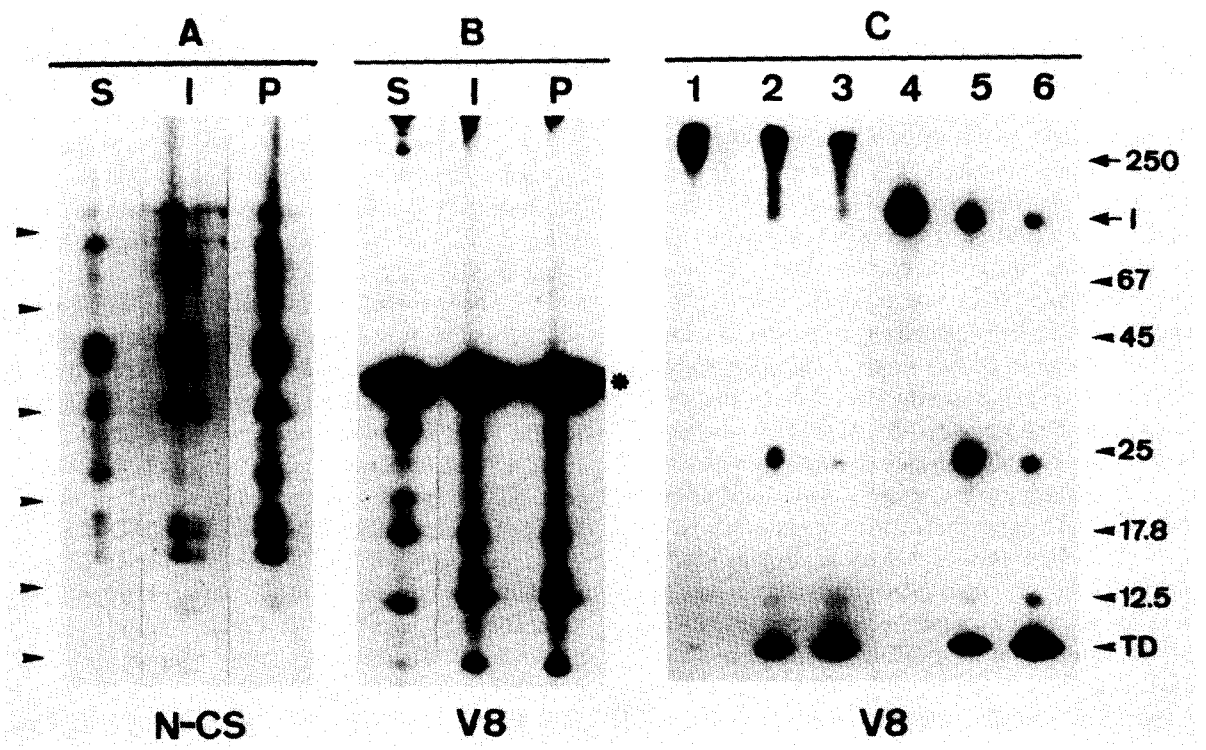


Fig. 6. Peptide mapping of the individual polypeptides of sucrase-isomaltase. The sucrase (S) and isomaltase (I) subunits and the 250 kDa protein (P) of the chicken sucrase-isomaltase preparation were isolated by SDS-PAGE, fragmented either with *N*-chlorosuccinimide (panel A) or with V8 protease (panels B and C) and fractionated by SDS-PAGE (13% acrylamide) as described in Materials and Methods (5 μg of V8 protease were used in panel B; 0, 2, and 5 μg for lanes 1 and 4, 2 and 5, and 3 and 6, respectively, in panel C). In panels A and B the silver-stained gels are shown. In panel C the [^{125}I]TID-labeled fragments produced from the 250 kDa protein (lanes 1–3) and from isomaltase (lanes 4–6) are visualized by autoradiography. The marker proteins (bovine serum albumin, ovalbumin, chymotrypsinogen A, myoglobin, cytochrome *c*) are indicated with the respective molecular weights in kDa. The arrows on the left refer to panels A and B, those on the right to panel C.

citraconylation (see Methods) of native preparations of sucrase-isomaltase did not lead to dissociation or disappearance of the 250 kDa band.

Discussion

The sucrase-isomaltase complex

The sucrase-isomaltase complex is one of the major intrinsic proteins of the small-intestinal brush-border membrane. By studies mainly with the rabbit enzyme, but also with that from hog, rat and man (reviewed in Refs. 2, 3, 32), it has been established that in mammals the mature sucrase-isomaltase complex is composed of two glycosylated subunits carrying a single catalytic site each. The smaller subunit, sucrase (approx. 140 kDa, apparent molecular weight on SDS-PAGE), does not interact directly with the lipid bilayer, but is non-covalently attached to isomaltase (approx. 160 kDa), which itself is anchored in the membrane by a hydrophobic segment located at its amino terminus [10,33]. This anchor peptide spans the bilayer once with the amino terminus being located on the cytosolic side of the brush-border membrane [34]. The sucrase-isomaltase heterodimer dimerizes further [35].

In order to explain in a single framework the many similarities (and thus presumed homology) of the subunits, their related biological control, and the mode of anchoring of the complex in the membrane, the 'one-chain, two-active-site hypothesis' had been proposed in 1977/78 [36–38, 1,2,32]. In the last years, a host of experimental data, most recently the cloning and sequencing of the cDNA encoding rabbit sucrase-isomaltase [34],

has fully confirmed this hypothesis. In summary, an ancestral gene coding for a one-polypeptide chain, one-active site enzyme splitting both maltose and isomaltose (a 'simple isomaltase') was partially duplicated resulting in a gene coding for a long, single polypeptide chain with two identical active sites (a 'double isomaltase'). Mutations changed one of these active sites from an isomaltase-maltase into a sucrase-maltase. Thus, a long polypeptide chain was formed, carrying two similar, but not identical active sites. Posttranslational modification of this single, long polypeptide chain by pancreatic proteinases, leads to the two subunits that make up the final sucrase-isomaltase complex. The subunits remain associated with one another via interactions formed during the folding of the original single chain pro-SI. (Interestingly, some mammalian species belonging to the Pinnipedia, like the sea lion (*Zalophus californianus*) have a long chain isomaltase with two identical catalytic sites, both splitting isomaltose and maltose, but not sucrose [39]. This double isomaltase thus mimicks the ancestral 'double isomaltase', but is likely to have originated by back mutation.)

The scope of the work presented here was to investigate the characteristics of an avian sucrase-isomaltase, in particular its subunit organization and its mode of anchoring and biosynthesis, in order to close some gaps in the phylogenetic pedigree of the sucrase-isomaltase complex and, possibly, to define more exactly the time of the gene duplication and of the emergence of sucrase activity.

Sucrase-isomaltase from chicken, isolated in its intact form by detergent solubilization, is composed of two large polypeptides with apparent molecular weights of 130 kDa and 140 kDa, very similar to the mammalian subunits of 140 kDa and 160 kDa. In contrast to all mammalian species studied, however, isomaltase activity is associated with the smaller of the two subunits and sucrase activity with the larger one. (Incidentally, this observation should be taken as a warning not to identify the subunits of sucrase-isomaltase of other species solely on the basis of their relative molecular weights.) But like mammalian sucrase-isomaltase, the chicken sucrase-isomaltase complex is anchored in the membrane exclusively by the isomaltase subunit. This is demonstrated by experiments with the hydrophobic photoreagent [^{125}I]TID which selectively labeled isomaltase but not sucrase from within the lipid phase of the bilayer. The labeled anchor peptide produced by papain digestion of reconstituted sucrase-isomaltase/phospholipid proteoliposomes is very similar in size to that obtained from rabbit sucrase-isomaltase.

All preparations of detergent-solubilized sucrase-isomaltase were found to contain small amounts of a polypeptide with an apparent molecular weight of 250 kDa, corresponding fairly well to the sum of the molecular weights of the subunits of final sucrase-isomaltase. This large protein was shown to be the unprocessed precursor form of sucrase-isomaltase, pro-SI, since the 250 kDa polypeptide and final sucrase-isomaltase yielded very similar peptide patterns upon fragmentation by either *N*-chlorosuccinimide or V8 protease. Furthermore, the pattern of the [^{125}I]TID-labeled fragments produced from the 250 kDa protein and from isomaltase by V8 protease digestion were identical. Finally, like rabbit and hog pro-SI [30,31,40], the chicken 250 kDa protein is very sensitive to cleavage by elastase which produces two fragments indistinguishable from final sucrase-isomaltase on SDS-PAGE.

Pro-sucrase-isomaltase (pro-SI) co-purifying with sucrase-isomaltase either originates from contaminating intracellular membranes in the brush-border vesicle preparation or it indicates that proteolytic conversion of pro-SI to final sucrase-isomaltase in vivo is incomplete.

These results show that chicken sucrase-isomaltase as to its membrane anchoring, its precursor form, and, therefore, its mechanism of biosynthesis is very similar to the mammalian sucrase-isomaltases. The (minor) difference in processing of pro-SI to final sucrase-isomaltase which in chicken leads to an isomaltase subunit that is smaller than the sucrase subunit, rather than larger, has probably no further implications. The site(s) of cleavage of rabbit pro-SI lies well within the 'sucrase domain', as judged by the internal homology in the sequence of pro-SI, so that a segment of the 'sucrase domain' becomes the carboxy terminal portion of the final isomaltase subunit [34].

The very close similarity between mammalian and avian sucrase-isomaltase strongly indicate that the duplication of the ancestral isomaltase gene and the evolution of sucrase by mutation of the distal gene segment took place prior to the separation of the progenitors of mammals from those of the birds and reptiles, i.e. more than 300 million years ago. If this is so, one should predict that reptilian sucrase-isomaltase should also be very similar to the mammalian enzyme. As yet we have no structural data about reptilian sucrase-isomaltase. However, it is known that turtle sucrase has kinetic characteristics (such as the non-essential activation by Na^+, which has no obvious biological significance [41]) in common with sucrase from mammals and chicken.

There are very few studies on the intestinal glycosidase activities in amphibia. They suggest that amphibia, in contrast to all other groups of terrestrian vertebrates, lack sucrase activity (reviewed in Ref. 42). The events leading to the appearance of this enzyme are thus likely to have occurred after the separation of amphibia from the other vertebrates, i.e. more recently than 350 million years ago. Clearly, more information on amphibian glycosidases is in want, in particular on the properties of their α-1,6-glucosidase(s).

The maltase-glucoamylase complex

Chicken glucoamylase consists of two subunits of apparent molecular weights very similar to those of sucrase-isomaltase. But unlike sucrase-isomaltase, it is the larger subunit which anchors the complex in the membranes, as was shown by [^{125}I]TID-labeling experiments *. Thus, for studies of either enzyme, it is essential to avoid cross-contamination by the other.

The glucoamylase preparations also contained a protein of approximately 250 kDa, but in quantities that were insufficient to perform a similar study as the one carried out for the 250 kDa protein of sucrase-isomaltase. However, pigeon intestinal glucoamylase has been described as a 260 kDa protein [8], and mammalian glucoamylase is also synthesized as a high molecular weight precursor which is only partially processed [43]. These observations make it very likely that the 250 kDa protein of chicken glucoamylase preparations is pro-glucoamylase.

Recent data on hog glucoamylase have localized the hydrophobic anchor segment at the amino terminus of one of the subuntis [44]. All these similarities with the sucrase-isomaltase complex suggest that the characteristics of the sucrase-isomaltase system (mammalian or avian) also apply to the glucoamylase system.

* The glucoamylase from rat intestine is also anchored to the membrane via the larger subunit [45].

Acknowledgments

We thank Dr. J. Brunner, Zürich, for his valuable help and advice, as well as for providing the photoreagent TID, Drs. H. Sjöström and O. Norén, Copenhagen, and Dr. H. Wacker, Zürich, for providing rabbit anti-hog SI and goat a. guinea pig anti-rabbit SI sera, respectively. Our work was partially supported by the SNSF, Berne, and by Nestlé Alimentana, Vevey.

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