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**Original article**

# Permeability of milk protein antigens across the intestinal epithelium *in vitro*

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**Summary** — Degradations by proteolytic enzymes and intestinal epithelial permeability represent two major drawbacks to the transfer of food protein antigens to blood. These steps were studied *in vitro* for the milk protein antigens β-Lactoglobulin (β-Lg), α-Lactalbumin (α-La) and β-casein (β-cas). Pepsin-trypsin hydrolysis and permeability in isolated rabbit ileum in Ussing chamber were suited by ELISA and radiolabelled-protein measurement. Pepsin-trypsin hydrolysis showed an increasing resistance in the order β-cas < α-La < β-Lg. The rate of absorption of the antigenic proteins by isolated rabbit ileum was in the same order, and the rate of absorption of the whole proteins (degraded and antigenic forms) was significantly higher for β-Lg than for α-La and β-cas. These results suggest a selective intestinal permeability for milk protein antigens. This selectivity is probably important in the mechanism of food protein sensitization via the oral route.

**absorption — intestine — β-Lactoglobulin — α–Lactalbumin — caseins**

**Résumé — Absorption intestinale *in vitro* des protéines de lait.** *La digestion par les protéases intraluminales et la perméabilité de l'épithélium intestinal représentent deux étapes limitantes majeures pour le transfert d'antigènes protéiques alimentaires vers le sang. Ces étapes ont été étudiées* in vitro *dans le cas de la β-Lactoglobuline (β-Lg), l'α-Lactalbumine (α-La), et la β-caséine (β-cas) du lait de vache. L'hydrolyse successive pepsine-trypsine, et la perméabilité à travers l'iléon de lapin isolé en chambre de Ussing, ont été suivies par dosage ELISA et par mesure radioisotopique. L'hydrolyse pepsine-trypsine indique une résistance croissante dans l'ordre β-cas < α-La < β-Lg. Les vitesses d'absorption des protéines sous forme antigénique se rangent dans le même ordre, et la vitesse d'absorption totale (forme antigénique et dégradée) est significativement plus élevée pour la β-Lg que pour l'α-La et la β-cas. Ces résultats suggèrent une perméabilité sélective de l'intestin pour les antigènes protéiques du lait. Cette sélectivité joue probablement un rôle important dans les mécanismes de sensibilisation par voie orale.*

***absorption — intestin — β-Lactoglobuline — α-Lactalbumine — caséines***

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## INTRODUCTION

The mechanism inducing food protein sensitivity via the oral route is complex and still unclear (Patrick & Gall, 1988). It is generally accepted that proteins or their antigenic fragments, which resist degradation by proteolytic enzymes of the gastrointestinal tract and cross the intestinal mucosa intact, are able to stimulate immunocomponent cells in the lamina propria (Baird *et al.*, 1987; Fällström *et al.*, 1984; Cornell *et al.*, 1971; Heyman *et al.*, 1982; Koritz *et al.*, 1987; Stern & Walker, 1984; Walker *et al.*, 1972). For this reason, it is important to know the proportion of antigenic protein that remains present during digestion by gastrointestinal enzymes and during their intestinal transepithelial passage. For a study of this kind, milk protein antigens appeared to be a good model since they are known to induce anaphylactic sensitization in some subjects (Baird *et al.*, 1987; Changin *et al.*, 1981; Huang *et al.*, 1985; Pearson *et al.*, 1983; Suzuki *et al.*, 1987) and are easily available in a pure form (Mercier *et al.*, 1968).

In the present study, the bovine milk protein antigens β-Lactoglobulin (β-Lg), α-Lactalbumin (α-La) and β-casein (β-cas) were suited by immunoenzymatic assay (ELISA) or radiolabelled-protein measurement during pepsin-trypsin hydrolysis *in vitro*, and during their transepithelial passage through isolated rabbit ileum. The results showed that β-Lg antigens were more able to resist pepsin-trypsin degradation and to cross the intestinal mucosa.

## MATERIALS AND METHODS

### *Chemicals*

Bovine β-Lg and α-La were of commercial origin (Sigma) and β-cas was purified from fresh milk (Mercier *et al.*, 1968). Proteins were $^{14}C$-radiolabelled by reductive alkylation of amino groups using $^{14}C$-formaldehyde (Jentoft & Dearborn, 1979).

### *Pepsin-trypsin hydrolysis*

Successive pepsin-trypsin (E/S – 1/100) hydrolysis of the proteins (5 mg/ml) was performed at 37 °C at pH 2 adjusted with HCl for pepsin, and in 0.02 M sodium borate pH 8.2 for trypsin. Aliquots were collected at different times for free α-amino group determination (Reimerdes & Klostermeyer, 1976), and ELISA.

### *Using chamber experiments (Powell* et al., *1972)*

White, male, New Zealand rabbits weighing 2–3 kg were killed by intravenous pentobarbital sodium injection. Pieces of stripped distal ileum were mounted between the two halves of a Lucite chamber (exposed area 3.14 $cm^2$) and bathed on each side at a temperature of 37 °C by 12 ml of isotonic Ringer solution containing (in mM) 140 $Na^+$, 5.2 $K^+$, 1.2 $Ca^{2+}$, 1.2 $Mg^{2+}$, 120 $Cl^-$, 25 $HCO_3^-$, 2.4 $HPO^{2-}_4$, 0.4 $H_2PO_4^-$ (pH 7.4 with 95% $O_2$, 5% $CO_2$). The spontaneous transmucosal electrical potential difference (PD) was continuously short-circuited by a short-circuit current (Isc) delivered by an automatic voltage clamp system (WPI, New Haven, CT, USA) that corrected for fluid resistance. The electrical conductance (G) of the tissue was calculated according to Ohm's law. After 30 min, $^{14}C$-proteins were added to the mucosal reservoir (5 μCi, 1 mg/ml). Aliquots were collected when necessary from the mucosal and serosal reservoir for radioactivity measurement and ELISA. After 140 min, each reservoir was emptied and the corresponding solution adjusted to 12% trichloroacetic acid and centrifuged. The precipitate was used for HPLC analysis.

### *Analytical techniques*

For the enzyme-linked immunosorbent assay (ELISA), Microtiter plates (Polylabo) were coat-

ed overnight at 4 °C with 100 µl of samples adjusted to pH 9.6 with 0.5 M carbonate buffer. The plates were washed and incubated 1 h at 37 °C with 1% gelatin; after washing, they were incubated 1 h at 37 °C with 100 µl of the appropriate rabbit antiserum (1/1000). Detection was performed with goat anti-rabbit IgG conjugated with horseradish peroxydase (Institut Pasteur, 1/3000), and diaminoor thophenylene and $H_2O_2$ as substrates. The detection limit was 4 ng/ml for β-Lg and α-La, and 15 ng/ml for β-cas. High-pressure liquid chromatography (HPLC) was performed with a Waters gradient system equipped with a C18 µ-Bondapack column (350 x 4.6 mm), eluted at 40 °C with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 2 ml/min in 20 min.

### *Calculations*

Results were expressed as means ± SE. Statistical analysis was performed using the paired or unpaired Student's *t*-test.

## RESULTS

During pepsin-trypsin hydrolysis, β-cas released more free amino groups than α-La, whereas, β-Lg was the least degraded (Fig. 1A). After pepsin treatment, no antigenic response was detected for β-cas, whereas, 0.2 and 12% of α-La and β-Lg, respectively, continued to respond. The subsequent action of trypsin suppressed the antigenic reaction of α-La, but 1% of the response of β-Lg was still detectable after 180 min (Fig. 1B).

$^{14}$C-radiolabelled proteins were introduced on the mucosal side of rabbit ileum in an Ussing chamber (Table I). No effect was observed on the electrical parameters of the tissue. On the mucosal reservoir, the total radioactivity was not affected after 120 min of incubation, whereas, the antigenic response, that was not altered for β-Lg and α-La, was 20% reduced for β-cas after 120 min, indicating a slight degradation of β-cas in the mucosal reservoir.

After the introduction of the labelled proteins on the mucosal side of rabbit ileum, the serosal accumulated radiolabelled material increased with time for the three proteins, the steady-state being reached only after a 60-70 min period (Fig. 2). For β-cas, the serosal antigenicity was never different from that measured in a control chamber without mucosal proteins, whereas, with β-Lg and α-La, the antigenic response became significantly different from that of the

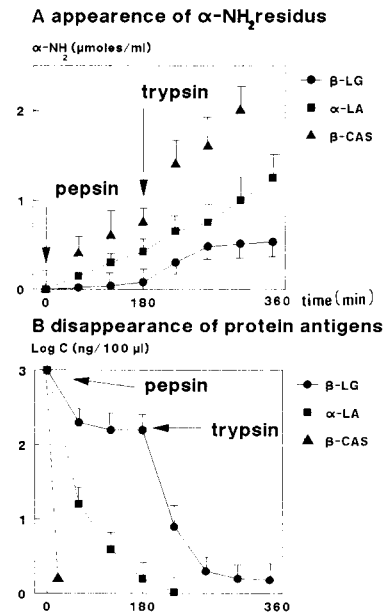


**Fig. 1.** Successive hydrolysis by pepsin and trypsin of β-Lg, α-La and β-cas. **A.** Release of free α-amino groups expressed in leucine equivalents (µmols/ml). **B.** Disappearance of antigenic determinants measured by ELISA and expressed in protein equivalents (mg/ml). Pepsin was introduced at t = 0, and trypsin at t = 180 min. Values are means ± SE for 4 experiments.

control chamber without mucosal proteins after 60 min and then linearly increased with time (Fig. 3). The rate of serosal accumulation of total radiolabelled material and antigenic proteins was then calculated in the 70-140 min period of incubation (Table II). The rate of total radiolabelled material accumulation was significantly higher with β-Lg than with α-La and β-cas. The rate of antigenic protein accumulation was significantly higher with β-Lg than with α-La and not detected for β-cas.

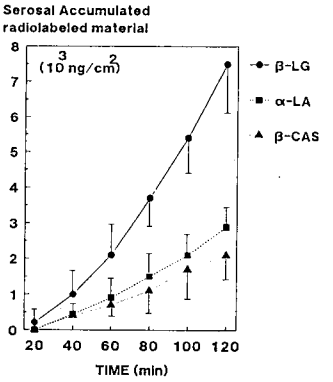


**Fig. 2.** Time-dependent accumulation of radioactivity on the serosal side of stripped rabbit ileum mounted in an Ussing chamber (exposed area 3.14 cm²), after the introduction into the mucosal reservoir of 1 mg/ml $^{14}$C-radiolabelled β-Lg, α-La or β-cas. Results are expressed in $^{14}$C-protein equivalents in ng/cm² of tissue. Values are means ± SE for 6 animals.

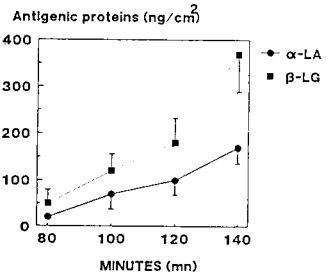


**Fig. 3.** Time dependent accumulation of antigenic determinants of α-La and β-Lg in the serosal side of stripped rabbit ileum mounted in an Ussing chamber (exposed area 3.14 cm²), after the introduction into the mucosal reservoir of 1 mg/ml $^{14}$C-radiolabelled β-Lg or α-La. Results are expressed in antigenic proteins in ng/cm² of tissue. Values are means ± SE for 6 animals.

At the end of the 140 min period of incubation of β-Lg or α-La on the mucosal side of rabbit ileum *in vitro*, the TCA-insoluble fraction of the serosal reservoir was analyzed by HPLC coupled with ELISA detection (Fig. 4). In each case, an antigenic response was detected exactly at the position of the intact proteins on the chromatogram.

## DISCUSSION

Cow's milk proteins are of high nutritional quality, but are known to induce hypersensitive reactions in some subjects (Changin *et al.*, 1981; Fällström *et al.*, 1984; Pearson *et al.*, 1983). These proteins consist of two fractions : a micellar casein fraction and a whey protein fraction. Bovine

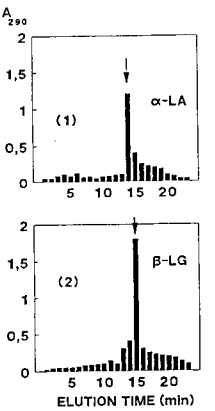


**Fig. 4.** Reverse-phase High Pressure Liquid Chromatography pattern with ELISA detection of the TCA-insoluble fraction of the serosal reservoir content of an Ussing chamber at time 140 min after introduction into the mucosal reservoir of 1 mg/ml α-La (1) or β-Lg (2).

**Table I.** Electrical parameters and mucosal concentration in β-Lg, α-La and β-cas, measured by $^{14}C$ counting or ELISA after introduction of 1mg/ml $^{14}C$-radiolabelled proteins, or Ringer only for control, in the mucosal side of rabbit ileum in an Ussing chamber.

| | | | *Ringer* | *β-Lg* | *α-La* | *β-cas* |
|---|---|---|---|---|---|---|
| Electrical Parameters | | Isc | 35.6 ± 4.7 | 36.2 ± 6.8 | 32.4 ± 2.5 | 42.7 ± 4.5 |
| | | PD | −2.3 ± 0.3 | − 2.3 ± 0.4 | −2.3 ± 0.2 | −2.4 ± 0.2 |
| | | G | 15.1 ± 1.7 | 15.9 ± 1.6 | 14.2 ± 1.6 | 14.7 ± 1.2 |
| Mucosal proteins (mg/ml) | t = 0 min | $^{14}C$ | — | 0.99 ± 0.05 | 1.03 ± 0.04 | 0.98 ± 0.05 |
| | | ELISA | — | 0.94 ± 0.09 | 0.97 ± 0.11 | 0.95 ± 0.09 |
| | t= 120 min | $^{14}C$ | — | 0.95 ± 0.06 | 0.92 ± 0.08 | 0.91 * ± 0.09 |
| | | ELISA | — | 0.90 ± 0.10 | 0.88 ± 0.09 | 0.69 * ± 0.09 |

Values are means ± SE for 8 animals. $^{14}C$-radiolabelled proteins (1mg/ml), or Ringer only for control, were introduced in the mucosal reservoir at time 0. Isc (μA/cm²), PD (mV) and G (mS/cm) are mean values in the 60-120 min period. * significantly different, $P < 0.01$. No significant difference was ever found for the electrical parameters.

casein is a mixture of the $\alpha s_1$, $\alpha s_2$, β and k caseins. The two major whey proteins are β-Lg and α-La. Caseins are demonstrated to be rapidly degraded by proteolytic enzymes of the gastrointestinal tract (Jakobson *et al.*, 1982; Miranda & Pelissier, 1981). The capacity for α-La digestion in the small intestine also seemed very large, whereas, β-Lg appeared the most resistant to luminal digestion (Fushiki *et al.*, 1986;

**Table II.** Transepithelial passage of β-Lg, α-La and β-cas in rabbit ileum mounted in an Ussing chamber *in vitro*.

| *Rate of serosal accumulation* ($\mu g.h^{-1}.cm^{-2}$) | *β-Lg* | *α-La* | *β-cas* |
|---|---|---|---|
| $^{14}C$-protein equivalents | 6.06 ± 1.14 | 1.92 ± 0.10 * | 1.89 ± 0.21 * |
| Antigenics proteins | 0.41 ± 0.03 | 0.13 ± 0.01 * | not detectable |

Values are means ± SE for 8 animals. Values are calculated from the linear part of the time-dependant serosal accumulation curves of $^{14}C$-protein equivalents (Fig. 2) and antigenic proteins (Fig. 3) in the 70-140 min period of incubation of the $^{14}C$-radiolabelled proteins (1 mg/ml) on the mucosal side of isolated rabbit ileum. * significantly different from β-Lg, $P < 0.05$.

Moritz *et al.*, 1987; Miranda & Pelissier, 1983). The present results confirm that β-Lg antigens are the most resistant to degradation by proteolytic enzymes. This lower degradability of β-Lg is probably linked to the structure of the protein which makes it less accessible to enzymes.

A main point of interest in this study is the evaluation of the intestinal permeability of milk proteins *in vitro*. Previous studies have established that proteins can be absorbed by the intestine and that the first stage is endocytotic uptake at the luminal membrane (Cornell *et al.*, 1971; Walker *et al.*, 1972). In the present study, this total uptake, evaluated as radiolabelled-protein equivalent transfer, exhibited differential kinetics for β-Lg, in comparison to α-La and β-cas. This result suggests a differential uptake mechanism for β-Lg, which remains to be elucidated. After internalization, the main fraction was degraded through the lysomal system, but another small fraction escapes this degradation and is found intact after crossing the mucosa (Heyman *et al.*, 1982; Stern & Walker, 1984). In the present work, this intact antigenic fraction, measured by ELISA, was also different for the three proteins since none were detected for β-cas, but significant amounts were found for β-Lg and α-La. This result suggests that the resistance of these proteins to intracellular degradation is also differential.

In conclusion, the present results indicate that resistance to proteolytic enzymes, uptake at the luminal membrane, and probably intracellular processing of the milk proteins tested, were differential. In every case β-Lg, antigens appeared to be the most resistant to degradation and the best able to cross the intestinal mucosa. These observations coincide with the idea that β-Lg is the main factor responsible for milk protein sensitization via the oral route (Huang *et al.*, 1985; Koritz *et al.*, 1987). Further studies are required to determine the mechanism that controls this intestinal permeability to food protein antigens.

## REFERENCES

Baird A.W., Barclay W.S., Balzer-Yost B.L. & Cuthbert A.W. (1987) Affinity purified immunoglobulin G transfers immediate hypersensitivity to Guinea pig colonic epithelium *in vitro*. *Gastroenterology* 92, 635-642

Changin G., Pattern R., Verkasalo J., Kuitinen P. & Avilathi E. (1981) Cow's milk intolerance. *Acta Pediat. Scandinavia* 10, 31-35

Cornell R., Walker W.A. & Isselbacher K.J. (1971) Small intestinal absorption of horseradish peroxidase : a cytochemical study. *Lab. Invest.* 25, 42-48

Fällström S.P., Ahlstedt S., Carlson B., Wettergren B. & Hanson I.A. (1984) Influence of breast feeding on the development of cow's milk proteins antibodies and the IgE level. *Int. Archs. Allergy Appl. Immun.* 75, 87-91

Fushiki T., Yamamoto N., Naeshiro I. & Iwai K. (1986) Digestion of α–Lactalbumin in rat gastrointestinal tracts. *Agric. Biol. Chem.* 50, 95-100

Heyman M., Ducroc R., Desjeux J.F. & Morgat J.L. (1982) Horseradish peroxidase transport across adult rabbit jejunum *in vitro*. *Am. J. Physiol.* 242 (*Gastrointest. Liver Physiol.* 16), G558-G564

Huang Q., Coleman J.W. & Stanworth D. (1985) Investigation of the allergenicity of β-Lactoglobulin and its cleavage fragments. Int. *Archs. Allergy Appl. Immun.* 78, 337-344

Jakobson I., Lindberg T. & Enedikson B. (1982) *In vitro* digestion of cow's milk proteins by duodenal juice from infants with various gastrointestinal disorders. *J. Pediatr. Gastroenterol. Nutr.* 1, 183-191

Jentoft N. & Dearborn D.G. (1979) Labelling of proteins by reductive methylation using sodium cyanoborohydride. *J. Biol. Chem.* 254, 4359-4365

Koritz T.N., Suzuki S. & Coombs R.R.A. (1987) Antigenic stimulation with proteins of cow's milk via the oral route in Guinea pigs and rats. I. Measurement of antigenically intact β-Lactoglobulin and casein in the gastrointestinal contents of duodenum, jejunum and ileum. *Int. Archs. Allergy Appl. Immun.* 82, 72-75

Mercier J.C., Maubois S.L., Poznanski B. & Ribadeau-Dumas B. (1968) Fractionnement préparatif des caséines de vache et de brebis par chromatographie sur DEAE-Cellulose en milieu urée et 2-mercaptoéthanol. *Bull. Soc. Chim. Biol.* 50, 521-530

Miranda G. & Pelissier J.P. (1981) *In vivo* studies on the digestion of bovine caseins in the rat stomach. *J. Dairy Res.* 48, 319-326

Miranda G. & Pelissier J.P. (1983) Kinetic study of *in vivo* digestion of bovine unheated skim-milk proteins in the rat stomach. *J. Dairy Res.* 50, 27-36

Patrick M.K. & Gall D.G. (1988) Protein intolerance and immunocyte and enterocyte interaction. *Pediatric. Clin. North Am.* 35, 17-34

Pearson J.P., Kingston D. & Shiner M. (1983) Antibody production to milk proteins in the jejunal mucosa of children with cow's milk protein intolerance. *Pediat. Res.* 17, 406-412

Powell D.W., Binder H.J. & Curran P.F. (1972) Electrolyte secretion by the guinea pig ileum *in vitro*. *Am. J. Physiol.* 223, (*Gastrointest. Liver Physiol.* 16), G531-G537

Reimerdes E.H. & Klostermeyer H. (1976) Determination of proteolytic activities on casein substrate. *In : Methods in Enzymology*, vol. XLV, Proteolytic enzymology Part B (Lorand L., ed.) Academic Press, New York, pp. 26-28

Stern M. & Walker W.A. (1984) Food proteins and gut mucosal barrier. I. Binding and uptake of cow's milk proteins by adult rat jejunum *in vitro*. *Am. J. Physiol.* 246 (*Gastrointest. Liver Physiol.* 16), G556-G562

Suzuki S., Koritz T.N. & Coombs R.R.A. (1987) Antigenic stimulation with proteins of cow's milk via the oral route in Guinea pigs and rats. II. Antibodies to β-Lactoglobulin secreted into the alimentary canal and serum. *Int. Archs. Allergy Appl. Immun.* 82, 76-82

Walker W.A., Cornell R., Davenport I.M. & Isselbacher K.T. (1972) Macromolecular absorption : mechanism of horseradish peroxidase uptake in adult and neonatal rat intestine. *J. Cell. Biol.* 54, 195-205