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# IMMUNOCHEMICAL CHARACTERISATION OF SOYABEAN GLYCININ FRAGMENTATION ESCAPING SMALL INTESTINAL DIGESTION IN PRE-RUMINANT CALVES.

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**Target Audience:** Biochemists, animal nutritionists, molecular biologists, veterinarians.

## ABSTRACT

**Attempts were made to precise some biochemical features of immunoreactive fragments of glycinin previously evidenced by ELISA in ileal digesta of calves fed a milk replacer containing antigenic soyabean. This was carried out by a panel of specific polyclonal antisera and monoclonal antibodies in immunoblotting after SDS-PAGE. Under reducing conditions, two major polypeptides of Mr 17,000 and 21,000 were observed, the latter corresponding to undigested basic (B) polypeptides of glycinin. Putative acidic (A) fragments were not directly evidenced under reducing conditions. Under non-reducing conditions, four bands were observed with Mr of around 28,000, 35,000, 38,000 and 42,000. The two lighter bands were identified as partially digested AB-type structures. Assuming B polypeptides to be rather intact (Mr 21,000), undigested A fragments in the digesta were estimated to have Mr of around 7,000, 14,000, 17,000 and 21,000. It was concluded that large fragments of glycinin escaped small intestinal digestion in the calf, and that structures of AB type and possibly quaternary associations were still present.**

Key words: Calf nutrition, soyabean globulin, digestion, SDS-PAGE, immunoblotting.

## DESCRIPTION OF PROBLEM

Soyabean protein is widely used as a cheap source of protein in nutrition, and has been used to replace milk protein in the diets of veal calves. However, performance of calves fed soyabean based milk substitute diets remain unsatisfactory even after the elimination of anti-nutritional factors (i.e. protease inhibitors and lectins) commonly found in legumes. This has been attributed to, among others the relative resistance of soyabean globulins, glycinin and β– conglycinin (which constitute about 80% of the total protein found in

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soyabean), to digestion by mammalian enzymes. Thus large fragments of these molecules which escape digestion are said to be involved in immunological mediated gut disturbances in the calf (due to their antigenicity), which impair with their effective utilisation (1,2).

Glycinin, (also called 11S globulin due to its sedimentation coefficient) has a Mr of about 360,000. It is theoretically made up of six subunits, each with Mr of about 60,000. Each subunit comprises one acidic (A) and one basic (B) polypeptides, with Mr about 40,000 and 20,000 respectively (3). These polypeptides are highly heterogeneous since up to 13 A and 11 B forms have been identified by two dimensional electrophoresis (4). Digestion studies *in vitro* have shown that the *'A'* polypeptides are hydrolysed faster than the *'B'* polypeptides, and pepsinolysis is reported to be faster than trypsin digestion (5,6). *'A'* polypeptides are reported to be degraded by pepsin to peptides of Mr below 16,000 (7), 12,000 (6) or even 7,000 (8).

There is paucity of information on the chemical nature of soyabean protein fragments that escape digestion *in vitro*. It has however been reported that up to 10% of ingested immunoreactive glycinin escaped digestion in the ileum of calves fed antigenic soyabean protein (9), but the chemical nature of these fragments had not been identified. The aim of the present work is therefore to provide further information on the biochemistry of the immunoreactive fragments of glycinin that escaped small intestinal digestion in the calf, as previously evidenced by enzyme-linked immunosorbent assay (ELISA) (9).

## MATERIAL AND METHODS

### Materials

All reagents including anti-rabbit IgG, anti-mouse IgG, anti-rat IgG, as well as substrates for immunoblotting assays (diaminobenzidine) were purchased from Sigma Chemical Co., France. Molecular weight standards kit was from Pharmacia, Sweden. Nitrocellulose membranes (Hybond-C super) were from Amersham International, U.K. Electrophoresis system model 6450 was from Atto-Touzard (Touzard & Matigon), France. Transblot apparatus for the transfer of proteins from gels to nitrocellulose membranes was from Pharmacia, Sweden.

### *In vitro* digestion studies

Detailed experimental procedure of this trial has already been published (9). Briefly, six preruminant Holstein calves were each fitted with an abomasal catheter and a re-entrant ileocaecal canula at the age of two months. They were fed three milk replacer diets for two weeks each, according to a double latin square designed experiment. Protein in diets was provided either by skim milk powder only (control), or by a mixture of skim milk powder (50% on a crude protein (CP) basis) and antegenic soyabean products (50% on CP basis). Ileal digesta were collected from canulae over 4 days during the second week of each experimetal period. Representatives samples were frozen at -20C and freeze at -20C and freeze-dried for analysis.

### *In vitro* immunochemical studies

#### Preparation of antibodies

Rabbit polyclonal antiserum (Pab) was prepared against purified native glycinin (JR J4) by injecting the latter subcutaneously to rabbits. The serum was collected after appropriate booster injections as already described (9)

Pab against SDS-denatured glycinin (Pab JR 9205) was prepared as follows:Glycinin polypeptides A and B were separated by sodium duodecil sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Slices of gel slabs containing *A* and *B*polypetitides were together injected subcutaneously to rabbits, and serumwas collected after appropriate booster injections (9,10). Pabs against *A* and *B* polypeptides (BTP 03P and BTP 02 respectively) of pea legumin (the 11S equivalent of glycinin) which cross-reacted in ELISA with corresponding *A* and *B*polypeptides of glycinin were obtained from Laboratoire de Biochimie et de Technologie des proteines, Nantes, France.

Monoclonal antibodies (Mabs) produced against an epitope of glycinin A (Mab IFRN 0025) (11) were obtained from the Department of Food Molecular Biochemistry, Institute of Food Research, Norwich, UK.

The properties of these Pabs and Mabs are summarised in Table 1.

#### Protein extraction and assay:

Protein from digesta of calves fed soyabean-based (antigenic and low antigenic) and the control diets was extracted in borate buffer for 1.5h at room temperature (RT) (9,12). Concentration of protein in solutions was determined by the method of Lowry (13), using bovine serum albumin as standard

SDS-PAGE and immunoblotting.

Electrohoresis (14) was carried out on mini-gels ( 80x 90m), in the presence of Trisglycine buffer (25 mM Tris, 192 mM glycine, pH 8.3) using 12.5 and 4% polyacrylamide for separating and staking gels, respectively. Amounts of protein deposited in the wells were 5 μ g for pure glycinin and 65μ g for digesta. Molecular weight standards were loaded in a separate well. Electrophoresis was performed for 1.5h at 40 mA, with or without dithiothreitol (200 mM) - reducing and non reducing conditions respectively. Electrophoresis was monitored using Coomassie blue staining.

After electrophoresis, proteins were electro-transferred (1 h, 100 mA) to nitrocellulose membranes in Tris - glycine buffer containing 0.1% SDS and 20% methanol. This transfer was monitored using Ponceau red staining. The membranes were then blocked using 5% skim milk powder in Tris buffer (20mM Tris, 37mM Nacl, pH 7.6) forl h at RT. The membranes were then incubated with optimal dillutions of specific antibodies (Table 1) overnight at RT. After washing, the membranes were incubated with appropriate horseradish-peroxidase conjugated antibodies diluted at 1:1000, for 2 h at RT. Finally, immuno-labelling was revealed by incubating the membranes with diaminobenzidine,(0.7mg/mL) for 5 to 10 minutes at RT.

**Table 1. Properties of antibodies used to characterise immunoreactive fragments of gycinin in digesta of calves.**

| Code name | Antigen used | Produced in: | Working dilution | Gycinin recognition |
|---|---|---|---|---|
| *Polyclonal antigen (Pabs)* | | | | |
| JR J4 | **Native glycinin** | **rabbit** | **1:5000** | **Acidic polypeptides mainly** |
| JR 9205 | SDS-denatured | rabbit | 1:5000 | Acidic and basic polypeptides |
| BTP 02 | Basic polypeptide of pea legumin | rabbit<br>rabbit | !:5000<br>1:5000 | Basic polypeptides |
| BTP 03 | Acidic polypeptide of pea legumin | rabbit | 1:64,000 | Acidic polypeptides |
| *Monoclonal antibodies (Mabs)* | | | | |
| IFRN 0025 | Subtisilin-treated glycinin | rat | 1:16,000 | Acidic polypeptides |

## RESULTS AND DISCUSSION

### Immunobloting after SDS-PAGE under reducing conditions

Pab JR J4 which was produced against native glycinin, recognised *A* polypeptides of purified glycinin but was unable to label glycinin fragments in digesta (data not shown). It should however be noted that this Pab, used earlier in ELISA, detected, immunoreactive fragments of glycinin in ileal digesta of calves fed antigenic soybean (9).

Pab Jr 9205 which was produced against SDS-denatured *A* and *B* polypeptides recognised these molecules from purified glycinin, and labelled two major polypeptides of Mr around 17,000 (GPI) and 21,000 (GP2) in the six ileal digesta corresponding to the antigenic soybean diet (Figure 1). No immunoreactivity was found in digesta when calves were fed control or low-antigenic soyabean diets.

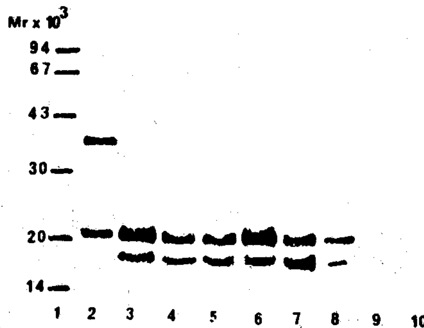


Figure 1. Immunobloting of glycinin and ilea digesta of calves using anti-SDS-denatured A and B polypeptides of glycinin (Pab JR 9205), after SDS-PAGE under reducing conditions.

Lane 1: molecular weight standards; lane 2 : glycinin; lane 3-8: digesta from calves fed antigenic soybean; lane 9: digesta from a calf fed low-antigenic soybean; lane 10:digesta from a calf fed skim milk powder.

Pab BTP 02 raised against *B* polypeptides of pea legumin cross-reacted well with glycinin *B* polypeptide (Figure 2). This Pab also bound to glycinin polypeptide GP2 in digesta. This indicates identity between GP2 and *B* polypeptide, but it does not exlude the presence of *A* fragments at that same Mr. Polypeptide GP1 was not labelled by Pab BTP 02, suggesting either the disappearance of B epitopes specific for this anitibody of the acidic origin of GP1. Surprisingly, Pab BTP 02 led to additional recognition of molecules of Mr around 43,000. Although not yet identified, this band was also present in a sample of control digesta (Figure 2) and did not appear to correspond to polypetides of B-conglycinin (data not shown).

BTP 03 raised against *A* polypetides of legumin cross-reacted nicely with glycinin A molecule. However, no specific labelling could be evidenced in ileal digesta of calves fed the antigenic soyabean diet. Similarly, Mab IFRN 0025 recognised glycinin *A* polypeptide of glycinin but did not recognise any fragments in ileal digesta (data not shown).

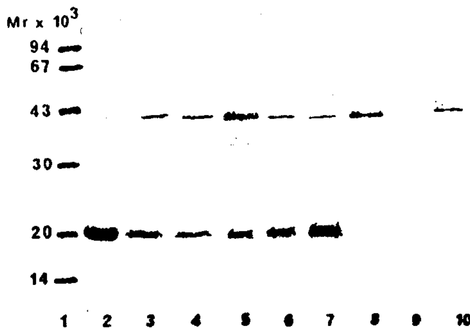


Figure 2. Immunobloting of glycinin and ilea digesta of calves using anti-*B* polypeptide of legumin (Pab BTP 02), after SDS-PAGE under reducing conditions. Lane 1: molecular weight standards; lane 2: glycinin; lanes 3-8 digesta from calves fed antigenic soybean; lane 9: digesta from a calf fed low-antigenic soybean; lane 10: digesta from a calffed skim milk powder.

## Immunoblotting after SDS-PAGE under non-reducing conditions.

Pab JR J4 recognised glycinin *AB* subunits (Mr 68000) but was still unable to detect any glycinin fragment in digesta. In fact, this Pab labelled one band in digesta only after non dissociating PAGE (data not shown).

Pab JR 9205 recognised nicely *AB* subunit of glycinin and a minor band of Mr around 42,000 ($A_4$ polypeptide?). Five out of the six ileal digesta collected in the antigenic soyabean group had immunoreactive peptides at Mr of 42,000 (minor band), and between 28,000 and 38,000 (major labeling corresponding to two-four bands (Figure 3). This indicates the presence in digesta of partially

digested *AB*-type structures. Labelling was not evidenced in lane 8, which corresponds to digesta from the calf that had the least amount of undigested immunoreactive glycinin as evidenced by ELISA (9).

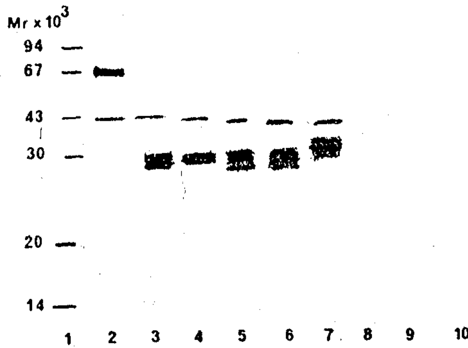


Figure 3. Immunobloting of glycinin and ileal digesta of calves using anti-SDS- denatured *A* and *B* polypeptides of glycinin (Pab JR 9205), after SDS-PAGE under non-reducing conditions. Lane 1: molecular weight standards; lane 2: glycinin; lanes 3-8: digesta from calves fed antigenic soyabean; lane 9; digesta from a calf fed low-antigenic soybean; lane 10: digesta from a calf fed skim milk powder.

Pab BTP 02 recognised only slightly AB subunit of glycinin and a minor component of Mr around 35,000 (Figure 4.) Most digesta from calves fed the antigenic soyabean diet exhibited labelling patterns with a major band of Mr around 35,000, and minor band at 28,000 in one calf. Another band of Mr around 43,000 was also evidenced, as already indicated for this antibody under reducing conditions.

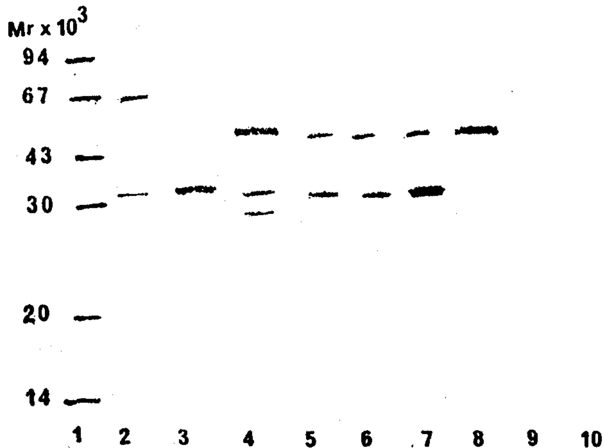


Figure 4. Immunobloting of glycinin and ileal digesta of calves using anti-*B* polypeptide of legumin (Pab BTP 02), after SDS-PAGE under non-reducing conditions. Lane 1: molecular weight standards; lane 2: glycinin; lanes 3-8: digesta from calves fed antigenic soyabean: lane 9: digesta from a calf low-antigenic soyabean; lane 10: digesta from a calf fed skim milk powder.

Mab IFRN 0025 strongly bound to *AB* subunit of glycinin (Mr 69,000) and to a minor component (Mr 35,000). Five out of six digesta collected in the antigenic soyabean group exhibited a strong labeling at Mr 35,000 (Figure 5). A faint band was also seen in two digesta samples at Mr around 28,000. This confirms the presence of A polypeptide fragments associated with *B* polypeptides in ileal digesta. It also suggests that the epitope recognised by Mab IFRN 0025 in digesta was related to the intergrity of AB- type structures.

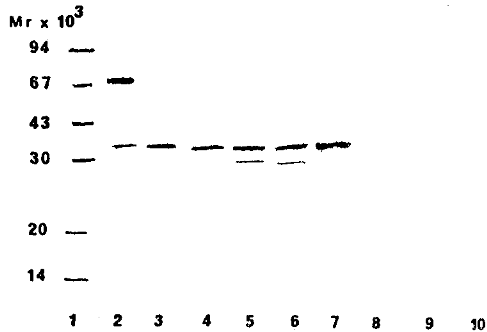


Figure 5. Immunoblotting of glycin and ileal digesta of calves using anti-*A* polypeptide of glycinin (Mab IFRN 0025), after SDS-PAGE under non-reducing conditions. Lane 1: molecular weight standards; lane 2: glycinin; lanes 3-8 digesta from calves fed antigenic soyabean; lane 9: digesta from a calf fed low-antigenic soyabean; lane 10: digesta from a calf fed skim milk powder

In this experiment, six immunoreactive fragments of glycinin have been directly or indirectly identified in ileal digesta of calves fed antigenic soyabean (Table 2). Under reducing conditions, polypeptides GPI (Mr 17,000) and GP2 (Mr 21,000) correspond to *B* (Sure for GP2) and possibly *A* fragments. Under non-reducing conditions, four peptides of Mr around 28,000 (GP3), 35,000 (GP4), 38,000 (GP5) and 42,000(GP6) have been evidenced. Two of them (GP3 and GP4) obviously derive from *AB* subunits of glycinin. Polypeptides GP5 and GP6 are probably *AB*-type structure too, rather than *A* polypeptides alone, since they were not observed under reducing conditions (Figures 1 and 2). *A* fragments indirectly evidenced here would therefore have possible Mr of 7,000 (AP1), 14,000 (AP2), 17,000 (AP3) and (or) 12,000 (AP4) (Table 3), if one consider that associated *B* fractions would be rather intact (Mr 21,000). The presence of AP3 and AP4 was strongly suggested from an antibody -exchange experiment in which anti- *A* and anti-*B* antibodies prepared ftom Pab JR 9205 recognised both GP1 and GP2 (data not shown). Bands GP5 and GP6 probably have an *AB*-type structure with possible intact B polypeptides and partially digested A polypeptides of Mr 17,000 and 21,000. However, when calculations are made using A polypeptide of Mr 17,000, one finds a B polypeptide of Mr 25,000 in the case of GP6, which is improbable (intact *B* polypeptides have Mr of 21,000). Therefore the question that still remains unanswered is the true origin of fragment GP1.

Table 2. Main features of glycinin polypeptides present in ileal digesta of calves fed antigenic soyabean, as recognised using various antibodies.
Antibody*

| Mr | Code name | Antibody | | |
|---|---|---|---|---|
| | | Pab JR 9205 | Pab BTP 02 | Mab IFRN 0025 |
| *SDS-PAGE under reducing conditions* | | | | |
| 17,000 | GPI | ++ | - | - |
| 21,000 | GP2 | ++ | ++ | - |
| *SDS-PAGE under non-reducing conditions* | | | | |
| 28,000 | GP3 | ++ | + | + |
| 35,000 | GP4 | ++ | ++ | ++ |
| 38,000 | GP5 | ++ | - | - |
| 42,000 | GP6 | | - | - |

*++ strong staining,+ medium staining,- no apparent staining, after immunoblotting

Our data indicate that a proportion of intact *B* polypeptides of glycinin can survive small intestinal digestion in the calf. However, the picture is less clear for *A* polypeptides which apparently seem to be more extensively digested. This would fit *in vitro* results which indicate that *A* polypeptides are more susceptible to pepsin, and to a lesser extent to trypsin digestion, than *B* polypeptides (5, 6, 15, 16).A polypeptides residues (after *in vitro* digestion) could have Mrs below 20,000 (5), 16,000 (7), 12,000 (6) or even 7,000 (8). It has been reported that *B* polypeptides pepsinolysis generated one band of Mr around 16,000, while leaving some *B* molecules intact, as evidenced by SDS-PAGE (6). Furthermore, two peptides of Mr 16,000 and 14,000 were obtained by peptic digestion of *A* polypeptides (6). Thus band GP1 (Mr 17,000) could have a double *A* and *B* origin.

Trypsinolysis of native glycinin *in vitro* for 30 minutes led to SDS-PAGE patterns of Mr estimated to be aound 31,000 (*A* fragment), 27,000 (*A* fragment), 21,000 (*B* intact), 17,000 (*A*?, *B*?), 15,500 (*A*?,*B*?) and 10,7000 (*A*?, *B*?) (6).

These data closely resemble those presented in Table 3, and suggest that suscestibility of glycinin to proteolysis is similar *in vitro* and *in vivo*. Glycinin polypeptides GP4 (Mr 35,000), identified as an *AB*-type structure, points to the presence of A fragments at the same Mr position. Indeed, an intermediate degradation product of Mr 33,000 was identified after *in vitro* trypsinolysis of both pea and soyabean 11S globulins (15, 16). Furthermore, using amino acid profile technique to analyse digesta from calves fed antigenic soyabean, it has been concluded that acidic polypeptides of glycinin, probably partially digested, might constitute an important part of soyabean protein escaping small intestinal digestion (17).

**Table 3: Determined and putative Mr of glycinin fragments present in ileal digesta of calves fed antigenic soyabean.***

| Code name | Determined Mr (origin) | Putative Mr of *A* or *B* fragments if: *A?* or *B?* = 17,000 | *A?* or *B?* = 21,000 |
|---|---|---|---|
| SDS-PAGE under reducing conditions | | | |
| GP1 | 17,000 (*A? B?*) | - | - |
| GP2 | 21,000 (*A? B*) | - | - |
| SDS-PAGE under non-reducing conditions | | | |
| GP3 | 28,000 (*AB, A?*) | 11,000 | 7,000 |
| GP4 | 35,000 (*AB, A?*) | 18,000 | 14,000 |
| GP5 | 38,000 (AB?, A?) | 21,000 | 17,000 |
| GP6 | 42,000 (AB?,A?) | 25,000 | 21,000 |

* A=acidic, B= basic, ?= possible origin.

As far as we know, the digestion of legume globulins *in vivo* has not been studied in depth. It has however been reported that undigested immunoreactive pea legumin (11S equivalent of glycinin) fragments detected at the ileum of calves had Mr of around 200,000 at 4h; and 70,000 and 40,000 at 8h post-feeding, as determined by gel filtration (12). The larger polypeptides, also found after *in vitro* proteolysis (15,16), could be a near-intact hexameric structure with partially digested A polypeptides. Indeed, the location of *A* polypeptides is thought to be superficial, with *B* polypeptides buried within the interior of the hexamer (11, 16). Preliminary work on non-dissocating PAGE of present ileal digesta indicated an immunoreactive glycinin band of high Mr. Thus, in vivo digestion steps of glycinin are probably similar to those of legumin, or the 11S globulins in general. These intermidiate products strongly reflect the complex but regular quaternary structure of native glycinin.

Glycinin is known to be highly immunogenic in calves and pigs (1 2). Attempts were made to find out whether plasma antibodies produced by our calves against antigenic dietary soyabean recognised glycinin fragments in digesta after SDS-PAGE. Whilst, these antibodies bound to intact *A* polypeptides, none of them recognised glycinin fragments in digesta. particularly *A* polypeptides, rather than B structures.

Ileal flow of glycinin determined by ELISA using Pab JR J4 has been estimated to be approximately 10% of immunoreactive glycinin intake (9). Surprisingly, this antibody recognised no glycinin fragments in digesta after SDS-PAGE, thus behaving like the calf antibodies.Thus flow of undigested glycinin in that experiment (9) might have been underestimated. This would partly explain discrepancies between that estimated flow (representing 0.2% of protein intake) and the flow of total soyabean protein (5% of protein intake) calculated from apparent ileal digestibility data. Therefore, in order to arrive at more precise estimates of soyabean protein digestion in the calf, global

quantitative digestion studies involving labelled animals (e.g $^{15}N$ technique) or dietary proteins (homoarginine technique) (18) may be required.

## CONCLUSIONS AND RECOMMENDATIONS

1 Results obtained here indicated that large fragments of glycinin, including intact *B* polypeptides, *AB*-type structures and even larger subunit assocaitions, escaped small intestinal digestion in the calf.
2 These observations are in broad agreement with digestion studies conducted *in vitro*, and suggest that the complex hexameric organisation of glycinin limits proteolysis by mammalian enzymes.
3 This may be responsible for the immunologically mediated hypersensitive reactions (the mechanisms of which are not yet fully understood) observed when calves are fed soyabean -based diets; since it is well known that undigested protein fragments in the small intestine could have antigenic effects in animals.
4 Further indentification of these fragments (e.g. by sequencing and possible identification of epitopes) could throw more light on the antigenic effects of soyabean proteins on calves. It could also open the way for developing varieties free of these epitopes by genetic engneering.

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