Study of the digestibility of yeasts, their cell walls and a mannoprotein preparation, Mannostab, by pancreatic enzymes.

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1. Introduction:

During alcoholic fermentation and maturing on lees, the yeast *Saccharomyces cerevisiae* releases mannoproteins into the wine. (Llauberes et *al.*,1988). These macromolecules constituting yeast cell walls (Ballou 1976) possess certain properties which are recognised in oenology. They are responsible in particular for improving wine stability, in regards to protein hazes (Ledoux et al. 1992) and tartaric acid salt crystallisation (Lubbers et al., 1994; Dubourdieu et Moine-Ledoux, 1995). These results have led us to the development of a mannoprotein extraction process (Dubourdieu and Moine-Ledoux, 1994) and to the industrial development of the new stabilisation product, Mannostab (Moine-Ledoux et Dubourdieu, 2002).

Wines stabilised with this new method could thus contain up to 300 mg/L of Mannostab. We consequently decided to investigate the digestibility of this mannoprotein preparation compared to the digestibility of entire yeasts or yeast cell walls.

This study on the digestibility of Saccharomyces cerevisiae yeasts, yeast cell walls and Mannostab has been carried out using the protocol n° CS/PM/2448 adopted by the SCF. This protocol allows the hydrolysis of polymer substances in solutions simulating digestive fluids to be measured (saliva, gastric fluid, pancreatic fluid).

The nature of substances to be digested, essentially macromolecules, and the results described in literature by Adrian et *al*, 1996 which demonstrate the high digestibility of yeasts and yeast cell walls in vivo, and which indicate that these substrates were preferentially hydrolysed by intestinal flora, have led us to eliminate the study of hydrolysis by solution simulating saliva or gastric fluid, and thus to concentrate on the effect of a solution simulating intestinal fluid.

In order to monitor hydrolysis of the yeasts and their cell walls; insoluble substances, we have chosen to measure the increase of solubilised substances in the reactive milieu by measuring the brix degree. For Mannostab, its hydrolysis is monitored using the gel filtration HPLC analysis method. (Moine-Ledoux et *al.*, 1997). This analysis is also carried out in trials with yeasts and yeast cell walls, in order to monitor whether their hydrolysis by pancreatic enzymes leads to mannoprotein release. Finally, the nitrogenous substances released during digestion by the intestinal fluid are monitored, using ninhydrin dosage (Rosen, 1957).

2. Materials and methods:

2.1. Digestion protocol:

Principle :

The substance to be digested is placed in the solution simulating digestive fluid, and maintained at 37°C with continuous agitation. After a specific action time of between 0,5 and 4 hours, the hydrolysis products or original substances are analysed in order to determine a hydrolysis percentage.

Products :

- Potassium hydrogenophosphate KH2PO4
- Sodium hydroxide NaOH
- Sodium taurocholate $C_{26}H_{44}NO_6SNa$ (SIGMA)
- Porcine pancreatic extract (SIGMA)
- Active dry yeasts (Laffort œnologie)
- Yeast cell walls (Fould springer)
- Mannostab (Laffort œnologie)
- Ninhydrin (SIGMA)

Solution simulating intestinal fluid:

Dissolution of 6,8 g of KH2PO4 in 250 ml of distilled water, transferred to a 1L vial, addition of 190 ml NaOH 0,2M, addition of 400 ml of distilled water, homogenisation. Dissolve 10 g of pancreatic extract in 150 ml of distilled water, when the extract is homogenous, add it to the vial. Add 0,5 g of sodium taurocholate and homogenise. The pH of the solution is adjusted to 7,5+/- 0,1 with NaOH 0,2M, the final volume adjusted to 1L.

Tests on the digestibility of yeasts and yeast cell walls by monitoring the brix degree measurement:

1 g of active dry yeasts (ADY) or 1g of yeast cell walls (YCW) is placed in 50ml of the solution simulating intestinal fluid at 37°C. A control test (Control) only containing 50 ml of the solution simulating intestinal fluid is placed at 37°C. The tests are carried out in triplicate.

Sampling is carried out at T = 0, 1, 2, 3 hours, sample size is 1 ml. The sample is centrifuged in order to eliminate insoluble matter, the brix degree measurement (°brix = g/100 mL) is carried out on the supernatant using an ATAGO hand refractometer type N1.

Tests for the digestibility of yeasts, yeast cell walls and Mannostab using gel filtration HPLC of released or hydrolysed mannoproteins:

250 mg of active dry yeasts (ADY) or 250 mg of yeast cell walls (YCW) or 250 mg of Mannostab (Msb) are placed in 50 ml of solution simulating intestinal fluid at 37°C. A control test (Control) only containing 50 ml of the solution simulating intestinal fluid is placed at 37°C. The tests are carried out in triplicate.

An initial sample is taken at T = 0, sample size is 2 ml. The sample is placed at 100°C for 1 minute in order to block enzymatic reactions, then centrifuged to eliminate insoluble matter, then frozen for subsequent analysis by gel filtration HPLC.

After 3 or 4 hours of digestibility, a second and third sample is taken for each modality in the same conditions as for the initial sample.

Mannoprotein dosage by HPLC gel filtration (Moine-Ledoux et al., 1997)

The mannoproteins are separated by gel filtration high pressure liquid chromatography on two stainless steel columns connected in series. The first column (0,75 x 7,5 cm), conditioned with trisacryl gel GF05 (IBF), permits the molecules to be separated by size exclusion chromatography. This gel with an exclusion molecular mass of 3 000 Da is normally used for desalting in low pressure chromatography, but its high mechanical resistance allows it to endure pressures of around 10 bars. The second column, (0,75 x 60 cm) containing TSK gel G2000 (LKB), is a gel filtration analytical column. The column's exclusion weight is 70 000 Da for globular proteins. The macromolecules are thus separated from other components by size exclusion chromatography for the first column and by gel filtration chromatography for the second column. The analysis conditions are the following: injected volume, 200 µl; eluent, NaCl 0,1 M ; flow rate, 0,6 ml/h (2150 HPLC pump); pressure 10 bars; recording speed, 0,5 mm/min (2210 recorder). The mannoproteins are detected by spectrophotometry at 220 nm (2158 Uvicrod Sd), calibration and identification are carried out in comparison with retention time and with those of the references obtained from extracted purified mannoproteins according to the method described by Moine-Ledoux et al, 1997.

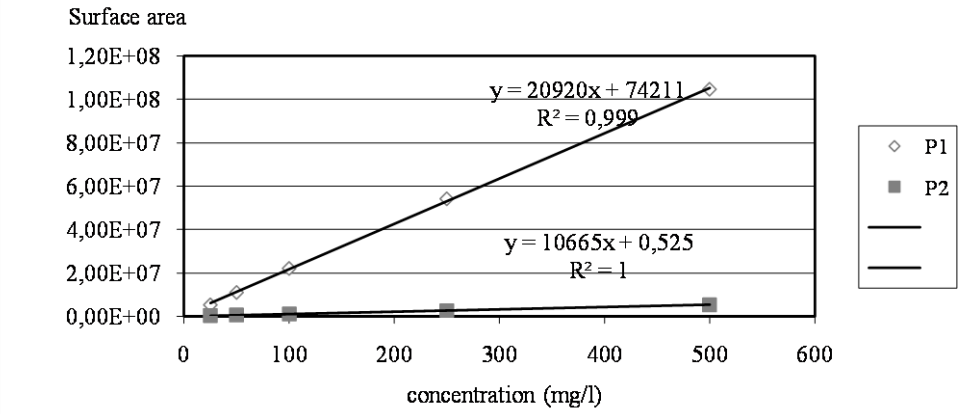

Figure 1 : Calibration of mannoprotein dosage (P1 et P2) by gel filtration HPLC.

Tests for the digestibility of yeasts, yeast cell walls and Mannostab by measuring the release of nitrogenous substances dosed with ninhydrin (Rosen, 1957):

250 mg of active dry yeasts (ADY) or 250 mg of yeast cell walls (YCW) or 250 mg of Mannostab (Msb)) are placed in 50 ml of solution simulating intestinal fluid at 37°C. A control test (Control) only containing 50 ml of the solution simulating intestinal fluid is placed at 37°C. Negative tests are carried out on the ADY, YCW, Msb and the control in the absence of pancreatic extract. All the tests are carried out in duplicate.

The nitrogenous substances are dosed at T= 0, 2 and 3 hours using the ninhydrin reactant.

Ninhydrin dosage of the nitrogenous substances :

1 ml of solution to be dosed (the sample having firstly been diluted 1/500) and 0,5 ml of the ninhydrin reactant (SIGMA) are placed in screw tubes. After 10 minutes in a water bath at 100°C, 2,5 ml of absolute ethanol was added to the cooled tubes. The formed violet-coloured complex is detected by colorimetry at 570 nm. Calibration is carried out using alanine solutions with concentrations of 6,4 µM to 250 µM (figure 2).

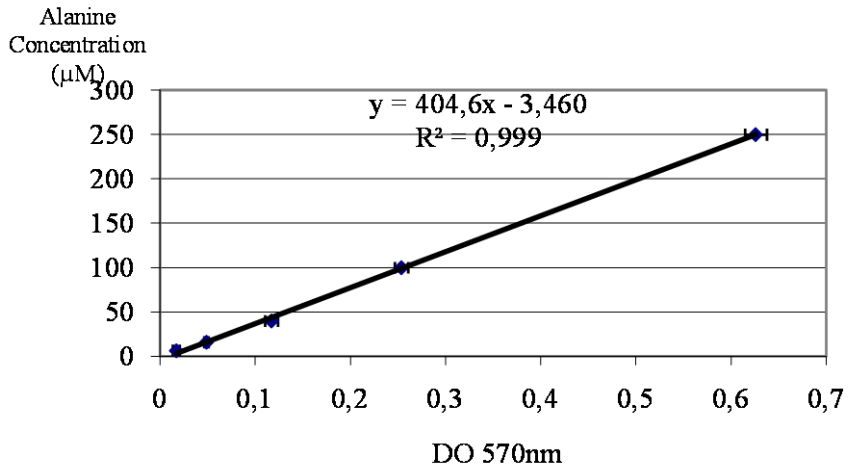

Figure 2 : Calibration of nitrogenous substance dosage using the ninhydrin method.

3. Results of hydrolysis by the solution simulating intestinal fluid of the yeasts, yeast cell walls and Mannostab

The results of the different analyses carried out are synthesised in the following tables. Individual data is presented in the appendix.

The yeast and yeast cell wall hydrolysis was monitored firstly by measuring the increase in soluble matter, using the brix degree in the reactive milieu. Results are presented in table I.

During digestion of entire yeasts or yeast cell walls, an increase in soluble matter in the reactive milieu can be noted. The pancreatic enzymes are therefore capable of hydrolyzing these two substrates.

Table I : Evolution of the brix degree during hydrolysis of yeasts and yeast cell walls in the solution simulating intestinal fluid.
The results are expressed by their average followed by their standard deviation (n=3)

Sample	Time (hours)				
	0	1	2	3	4
Control	4,2 ± 0.0	4,2 ± 0.0	4,2 ± 0.0	4,2 ± 0.0	4,2 ± 0.0
Active dry yeasts	4,2 ± 0.0	4,4 ± 0.0	4,5 ± 0.1	4,6 ± 0.0	4,7 ± 0,1
Yeast cell walls	4,4 ± 0.0	4,6 ± 0.0	4,7 ± 0.1	4,8 ± 0.0	4,9 ± 0.1

The digestibility of yeasts, yeast cell walls and Mannostab is also monitored using gel filtration HPLC in order to monitor whether their hydrolysis by pancreatic enzymes leads to mannoprotein release. Each sample at T = 0,3 and 4 hours is analysed by gel filtration HPLC in order to monitor the evolution of the mannoproteins separated in peaks 1 and 2 which correspond to molecules with a molecular mass superior to 70 Kda for peak 1 and to molecules between 50 and 40 Kda for peak 2 (table II).

Table II : Results of dosage by gel filtration HPLC of mannoproteins (P1 and P2, mg/L) during digestion of active dry yeasts, yeast cell walls and Mannostab by reconstituted digestive succus.

The results are expressed by their average followed by their standard deviation (n=3)

Sample	Time (hours)					
	0		3		4	
	P1	P2	P1	P2	P1	P2
Control	200 ±69	0 ± 0	160 ± 69	160 ± 69	120 ± 0	160 ± 139
Active dry yeasts	160 ± 69	0 ± 0	440 ± 277	320 ± 69	1840 ± 69	360 ± 0
Yeast cell walls	160 ± 69	0 ± 0	600 ± 208	280 ± 69	440 ± 250	200 ± 69
Mannostab	1200 ± 0	3680 ± 69	320 ± 69	760 ± 69	280 ± 69	680 ± 69

During hydrolysis by the solution simulating intestinal fluid, a high release of mannoproteins can be noted for yeasts, a smaller quantity for yeast cell walls, and a strong decrease during Mannostab hydrolysis.

The enzymatic complex of the intestinal fluid is essentially made up of amylases and proteases; the latter probably being mainly responsible for hydrolysing Mannostab, yeasts and yeast cell walls. We consequently decided to monitor the release of nitrogenous substances during a hydrolysis test in the presence (+ test) or the absence (- test) of pancreatic extract. These modalities are a means of showing whether the hydrolysis of the mannoproteins, yeasts or yeast cell walls is due to the pancreatic enzymes or to a simple autolysis phenomenon.

The results collected in table III show a strong release of nitrogenous substances during digestibility in the presence of pancreatic enzymes (+ test) and to a lesser extent by an autolysis phenomenon (- test).

Table III: Results of nitrogenous substance content (C) during digestion of dry yeasts, yeast cell walls and Mannostab by reconstituted intestinal fluids (+ : presence of pancreatic extract ; - : absence of pancreatic extract)

The results are expressed by their average followed by their standard deviation (n=3)

Samples	Time (hours)					
	0		2		3	
	+	-	+	-	+	-
Control	27588 ±832	83 ±48	26851 ±1902	273 ±48	29561 ±285	249 ±24
Active dry yeasts	31058 ±1022	1533 ±71	60751 ±3090	1937 ±48	72399 ±3994	2888 ±571
Yeast cell walls	33507 ±2615	772 ±71	36645 ±4517	820 ±24	53975 ±1498	1152 ±71
Mannostab	31772 ±404	392 ±24	40496 ±1854	986 ±48	60727 ±214	1818 ±71

The role of proteases in the hydrolysis of yeasts, cell walls and Mannostab by reconstituted intestinal fluids can be estimated by the concentration variation of the nitrogenous substances released during digestion (table IV). The interpretation of the results obtained clearly demonstrates that the release of nitrogenous substances during these digestibility tests is due to pancreatic enzymes and not only to an autolysis phenomenon. Yeast cell walls are the substances which among those tested are the least sensitive to autolysis.

Table IV : Interpretation of phenomena during the digestibility test of active dry yeasts, cell walls or Mannostab due to autolysis ((- test) – (control-)) or due to pancreatic enzymes ((+ test) – (control +) – autolysis).

Evolution of nitrogenous substances (mg/l)	Due to autolysis phenomena	Due to pancreatic enzymes
Active dry yeasts	1189	38179
Cell walls	214	18281
Mannostab	1260	25672

The digestibility assessments (table V) calculated using released substance dosages (total soluble matter, mannoproteins and nitrogenous substances) show the action of the pancreatic enzymes on the cell walls, the entire yeasts and the Mannostab. Yeast hydrolysis leads to the release of mannoproteins, essentially with high molecular weights. On the other hand, the pancreatic enzymes release few mannoproteins from cell walls. Moreover, we show that Mannostab is highly digestible by the solution simulating intestinal fluid at over 85%, and in relatively equal quantities for the two P1 and P2 fractions. Furthermore, we demonstrate that the pancreatic enzyme action is essentially due to the proteolytic activities which are at the origin of nitrogenous substance hydrolysis of yeasts, cell walls and Mannostab.

Table V : Output calculated according to the different digestibility evaluation methods (measurement of substances solubilised by the brix degree, mannoprotein dosage in gel filtration HPLC, nitrogenous substance dosage).

(C : control ; Msb : Mannostab ; YCW: yeast cell walls ; ADY : active dry yeasts)

			samples		
Evaluation methods			ADY	YCW	Msb
° Brix	0 hours	Dry weight (g/50ml)	1	1	-
	4 hours	Solubilised matter (g/50 ml)	0,265	0,235	-
	Output%	Sm/Dw	26,5 %	23,5 %	-
Mannoproteins	0 hour	DW : Dry weight mg/L	5000	5000	5000
		P1 mg/L	200	200	1200
		P2 mg/L	0	0	3680
		MP mg/L	200	200	4880
	4 hours	P1 mg/L	1720	320	160
		P2 mg/L	200	0	520
		MP mg/L	1920	320	680
	Output%	$(P1_4-P1_0)/ P1_0$	760	60	-87
		$(P2_4- P2_0)/P2_0$	100	0	-81
		$(MP_4- MP_0)/MP_0$	860	60	-86
		MP/DW	+ 34%	+ 2 %	- 84 %
Nitrogenous substances		DW : Dry weight mg/L	5000	5000	5000
	0 hour	Test(-)	1450	689	309
		Test (+)	3470	5919	4184
	3 hours	Test(-)	2639	832	1569
		Test(+)	42838	24414	31166
	% autolysed	$Test(-)_3-Test(-)_0/DW$	23,8 %	2,9 %	25,2 %
	% hydrolysed	$(Test(+)_3-Test(+)_0)-(Test(-)_3-Test(-)_0)/DW$	763 %	366 %	513 %

4. Discussion and conclusion

Our results show that yeasts or their cell walls, and Mannostab are hydrolysed by a solution simulating intestinal fluid. The work of Adrian et *al*, 1996 demonstrated the high digestibility of yeasts and yeast cell walls in vivo, and these authors ventured the hypothesis that these substrates were preferentially hydrolysed by intestinal flora. In fact, we demonstrate that pancreatic enzymes and in particular proteases are responsible for part of yeast and cell wall hydrolysis.

For Mannostab, a purified fraction of the cell wall, we demonstrate that this mannoprotein preparation is hydrolysable by pancreatic enzymes at over 80%.

4.1.1. Bibliographic references

Adrian J., Frangne R. et Potus J., 1996. Les parois des levures alimentaires et leurs incidences nutritionelles. Médecine et Nutrition, 4, 167-170.

Balou C. E., 1976.The structure and biosynthesis of mannan component of the yeast cell enveloppe. Adv. Microb. Physiol. 14, 93-158.

Dubourdieu D. et Moine-ledoux V., 1994. Produit biologique pour la stabilisation physico-chimique d'un vin. Brevet d'invention N° de publication 2 726 284.

Dubourdieu D. et Moine-ledoux V., 1995. In : *Oenologie 95.* Lavoisier TEC et DOC Ed, 385-390.

Ledoux V., Dulau L. et Dubourdieu D., 1992. Interprétation de l'amélioration de la stabilité protéique des vins au cours de l'élevage sur lies. J. Int. Sci. Vigne Vin, **26**,n°4, 239-251.

Llaubéres R. M., 1988. Les polysaccharides sécrétés dans les vins par *Saccharomyces cerevisiae et Pediococcus sp*., Thèse de doctorat es science, université de Bordeaux II.

Lubbers S., Léger B., Charpentier C. et Feuillat M., 1994. Effet colloïde-protecteur d'extraits de parois de levures sur la stabilité tartrique d'une solution modèle. J. Int. Sci. Vigne Vin, 27,n°1, 13-22.

Moine-Ledoux V., Perrin A., Paladin I. et Dubourdieu D., 1997. Premiers résultats de stabilisation tartrique des vins par addition de mannoprotéines purifiées (Mannostab ^{TM}). *J. Int. Sci. Vigne Vin*, 31 (1), 23-31.

Moine-Ledoux V. et Dubourdieu D., 2002. Une mannoprotéine à ancre GPI responsable de la stabilisation tartrique des vins. Mannostab : derniers développements industriels. R . F. OE, 193,32-35.

Rosen H., 1957. A modified ninhydrin colorimetric analysis for amino acids . Arch. Biochem. Biophys., 67, 102-115.

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Appendix I : Evolution of the brix degree during hydrolysis of yeasts and yeast cell walls in the solution simulating intestinal fluid.

(C :control ; Msb : Mannostab ; YCW : yeast cell walls ; ADY : active dry yeast)

Time (hours)	0	1	2	3	4
Modalities	° Brix	° Brix	° Brix	° Brix	° Brix
C 1	4,2	4,2	4,2	4,2	4,2
C 2	4,2	4,2	4,2	4,2	4,2
C 3	4,2	4,2	4,2	4,2	4,2
C average	**4,2**	**4,2**	**4,2**	**4,2**	**4,2**
C standard deviation	**0**	**0**	**0**	**0**	**0**
ADY 1	4,2	4,4	4,4	4,6	4,6
ADY 2	4,2	4,4	4,4	4,6	4,8
ADY 3	4,2	4,4	4,6	4,6	4,8
ADY average	**4,2**	**4,4**	**4,47**	**4,60**	**4,73**
ADY standard deviation	**0**	**0**	**0,12**	**0**	**0,12**
YCW 1	4,4	4,6	4,6	4,8	4,8
YCW 2	4,4	4,6	4,6	4,8	4,9
YCW 3	4,4	4,6	4,8	4,8	4,9
YCW average	**4,4**	**4,6**	**4,67**	**4,8**	**4,87**
YCW standard deviation	**0**	**0**	**0,12**	**0**	**0,06**

Appendix III : Results of mannoprotein dosage by gel filtration HPLC (P1 and P2, mg/L) during digestion of active dry yeasts, yeast cell walls and Mannostab by reconstituted digestive succus.

(C : control ; Msb : Mannostab ; YCW : yeast cell walls ; ADY :active dry yeast)

Time	0		3 hours		4 hours	
Modalities	P1	P2	P1	P2	P1	P2
C 1	240	0	120	240	120	0
C 2	120	0	120	120	120	240
C 3	240	0	240	120	120	240
C average	**200**	**0**	**160**	**160**	**120**	**160**
C standard deviation	**69**	**0**	**69**	**69**	**0**	**139**
ADY 1	120	0	120	240	1920	360
ADY 2	240	0	600	360	1800	360
ADY 3	120	0	600	360	1800	360
ADY average	**160**	**0**	**440**	**320**	**1840**	**360**
ADY standard deviation	**69**	**0**	**277**	**69**	**69**	**0**
YCW 1	120	0	840	360	240	120
YCW 2	120	0	480	240	360	240
YCW 3	240	0	480	240	720	240
YCW average	**160**	**0**	**600**	**280**	**440**	**200**
YCW standard deviation	**69**	**0**	**208**	**69**	**250**	**69**
Msb 1	1200	3720	360	840	360	720
Msb 2	1200	3600	240	720	240	720
Msb 3	1200	3720	360	720	240	600
Msb average	**1200**	**3680**	**320**	**760**	**280**	**680**
Msb standard deviation	**0**	**69**	**69**	**69**	**69**	**69**

Appendix IV: Evolution of nitrogenous substance content during digestion of dry yeasts, yeast cell walls and Mannostab by reconstituted intestinal fluids

(C : control ; M : Mannostab ; Y : yeast cell walls ; A :active dry yeast ; + : in presence of pancreatic extract ; - :without pancreatic extract)

	C(mg/l)			
time (hours)	0	2	3	C_3-C_0
C-	130	320	273	
C-	35	225	225	
C- avg	**83**	**273**	**249**	**166**
C- St Dev	48	48	24	
C+	28420	24949	29846	
C+	26756	28753	29276	
C+ avg	**27588**	**26851**	**29561**	**1973**
C+ St Dev	832	1902	285	
M-	368	938	1747	
M-	415	1034	1889	
M- avg	**392**	**986**	**1818**	**1426**
M- St Dev	24	48	71	
M+	31368	42351	60513	
M+	32176	38642	60941	
M+ avg	**31772**	**40496**	**60727**	**28955**
M+ St Dev	404	1854	214	
Y-	701	843	1224	
Y-	843	796	1081	
Y- avg	**772**	**820**	**1152**	**380**
Y- St Dev	71	24	71	
Y+	30892	41162	55473	
Y+	36122	32128	52478	
Y+ avg	**33507**	**36645**	**53975**	**20468**
Y+ St Dev	2615	4517	1498	
A-	1461	1984	2317	
A-	1604	1889	3458	
A- avg	**1533**	**1937**	**2888**	**1355**
A- St Dev	71	48	571	
A+	30036	57660	68405	
A+	32081	63841	76393	
A+ avg	**31058**	**60751**	**72399**	**41341**
A+ St Dev	1022	3090	3994	