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ORIGINAL PAPER

The non-nutritional performance characteristics of peptones made from rendered protein

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Abstract Economic considerations require the use of inexpensive feedstocks for the fermentative production of moderate-value products. Our previous work has shown that peptones capable of supporting the growth of various microorganisms can be produced from inexpensive animal proteins, including meat and bone meal, feather meal, and blood meal, through alkaline or enzymatic hydrolysis. In this work, we explore how these experimental peptones compare to commercial peptones in terms of performance characteristics other than chemical make-up; these characteristics can impact fermentation operating cost. It is shown that experimental peptone powders produced through enzymatic hydrolysis are highly hygroscopic and that their physical form is not stable to humid storage conditions; those produced through alkaline hydrolysis and commercial peptones are less hygroscopic. When used in growth medium, all peptones contribute haze to the solution; experiments show that the source of haze is different when using enzyme- versus alkali-hydrolyzed peptones. Alkali-hydrolyzed peptones and all peptones made from blood meal are stronger promoters of media foaming than the commercial peptones; some enzyme-hydrolyzed peptones support very little foam formation and are superior to the commercial peptones in this sense. Alkali-hydrolyzed peptones are roughly equivalent to commercial peptones in the coloration they contribute to media, while enzyme-hydrolyzed peptones contribute intense coloration to media. No peptone caused a significant change in the viscosity of media. The experimental peptones studied here may be acceptable low-cost substitutes for commercial peptones, but none is equivalent to the commercial products in all respects.

Keywords Peptone · Industrial fermentation · Rendered protein · Hydrolysate · Meat and bone meal

Introduction

Fermentation is an increasingly important method for producing commodity and moderate-value chemicals. Such fermentations differ from laboratory or pharmaceutical fermentations by being larger in volume and more sensitive to costs [1]. Operating costs for a fermentation process include the cost of growth medium ingredients, energy and labor, among others. Among growth media ingredients, peptones and similar substances such as yeast extract tend to be the most expensive components [2]. Our previous work has shown that peptones suitable for the growth media of some microorganisms can be produced from low-cost rendered proteins, including meat and bone meal, feather meal, and blood meal, through alkaline or enzymatic hydrolysis (manuscripts in preparation).

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In addition to meeting the nutritional requirements of microorganisms, there are other characteristics which affect the practical utility of a peptone. Highly hygroscopic peptone powders can be problematic for storage and conveyance; absorption of moisture reduces the chemical and microbial stability of the peptone and increases its cohesiveness, leading to caking and bridging. Peptone which is incompletely soluble or which precipitates out of growth medium during heat sterilization is undesirable because the undissolved portion may be less available to microorganisms and may tend to settle in the fermentor. A peptone that raises the viscosity or foaming tendency of the growth medium will increase energy consumption and the operational difficulty of a fermentor [3]. Peptones that contribute color or haze to the growth medium are often undesirable due to interferences with the real-time monitoring of the fermentation process and increased cost in downstream purification of the fermentation product.

We have produced experimental peptones from a variety of rendered protein types, hydrolyzing each with a variety of processes. In the present work we explore how these experimental peptones compare to commercial peptones in terms of non-nutritional performance characteristics.

Materials and methods

Rendered protein materials, hydrolysis reagents and commercial peptones

Ruminant meat and bone meal (MBM) and flash-dried cattle blood meal (BM) were obtained from Darling International (Irving, TX, USA); hydrolyzed feather meal (FM) was obtained from Carolina By-Products (Winchester, VA, USA). The hydrolytic agents included Bell Mine hydrated lime, high calcium (Tannin Corp., Peabody, MA, USA), Versazyme (BioResource International, Morrisville, NC, USA), Alcalase 2.4L and Flavourzyme (Novozymes, Bagsvaerd, Denmark). Commercial peptones used included Bacto Yeast Extract (BYE) and Bacto Typtone (BP) from Becton Dickinson and Company (Franklin Lakes, NJ, USA) and casamino acids (CA) from MP Biomedicals (Irvine, CA, USA).

Peptone production

The peptone production process is detailed in a separate publication (in preparation). Process variables included type of rendered protein substrate used and method of hydrolysis (Table 1). Briefly, the rendered protein was partially defatted by hexane extraction prior to hydrolysis. Hydrolysis was conducted in a thermostated reaction vessel with constant stirring adequate to keep the rendered protein in suspension. All reactions consisted of 9.1% (w/w) rendered protein in water. The alkaline hydrolysates were produced in 6 L batches, with 0.1 g CaOH/g substrate, at 85°C, for 4–16 h. The reaction was terminated by sparging with CO_2 until the pH dropped to 9, followed by neutralization with sulfuric acid. The enzymatic hydrolysates were produced in 4 L batches at 50°C. At the beginning of the reaction Alcalase or Versazyme was added; the pH was monitored continuously and maintained at the manufacturer's recommended pH through the addition of 8 M NaOH. In some reactions, after 4 h the pH was allowed to drop to 7.0 and Flavourzyme was added. Reactions were terminated by raising the reaction temperature to 90°C for 10 min. After either type of reaction, residual solid material was removed by centrifugation followed by filtration through a filtration capsule (Millipore, Billerica, MA, USA) with 0.45 μm pores. The remaining hydrolysate was dehydrated using a Büchi (Flawil, Switzerland) B-191 Mini Spray Drier.

Table 1 The range of experimental peptones produced and tested

Raw material	Hydrolysis method					
	Alkaline			Enzyme		
	4 h	8 h	16 h	V	A	A + F
MBM	x	x	x	x	x	x
FM	x	x	x	x	x	x
BM	x	x	x	x	x	x

Under "alkaline," *4 h*, etc. refers to the duration of raw material hydrolysis. Under "enzyme," *V* is Versazyme, *A* is Alcalase, and *F* is Flavourzyme. The *x*'s represent a set of conditions from which the experimental peptone was produced

Hygroscopicity

Relative hygroscopicity was determined by measuring the mass of water absorbed per gram of powdered peptone when equilibrated with 75.3% relative humidity air. Briefly, triplicate samples of approximately 1 g were dehydrated and the dry mass was determined. The samples were transferred to a constant relative humidity chamber, prepared according to ASTM E 104-02 [4] with NaCl, and incubated at 25°C, until they reached a constant mass (66 days).

Liquid media

Experimental peptones were used in place of commercial peptone in the preparation of two types of liquid growth media. Most experiments used a simple fungal growth medium [5] in which experimental peptone substituted for yeast extract (Table 2). Where specified, a bacterial growth

Table 2 The compositions of the media used

Bacterial medium	
pH	7.0–7.2
Glycerol	40 g/l
Casamino acids*	10 g/l
KH_2PO_4	5.55 g/l
$K_2HPO_4{\cdot}3H_2O$	5.60 g/l
$NaH_2PO_4{\cdot}H_2O$	2.25 g/l
Citric acid	3 g/l
$(NH4)_2SO_4$	1.00 g/l
NH_4Cl	0.10 g/l
$CaCl_2{\cdot}2H_2O$	20.00 mg/l
$MgCl_2{\cdot}6H_2O$	1.25 g/l
$MnSO_4{\cdot}H_2O$	12.90 mg/l
$CoCl_2{\cdot}6H_2O$	6.45 mg/l
$FeSO_4{\cdot}7H_2O$	0.02 mg/l
$ZnSO_4{\cdot}7H_2O$	8.70 mg/l
$CuCl_2{\cdot}2H_2O$	3.20 mg/l
$Na_2MoO_4{\cdot}2H_2O$	2.70 mg/l
$AlCl_3$	0.80 mg/l
H_3BO_3	0.50 mg/l
Thiamine–HCl	5.00 mg/l
Fungal medium	
pH	6
Glucose	20 g/l
Yeast extract*	10 g/l

* Components that were replaced with experimental or commercial peptones

medium [6], in which experimental peptone substituted for casamino acids, was used. All media were sterilized on a standard autoclave liquid cycle.

Chromicity

Chromicity was determined in triplicate and was measured using a 1 cm quartz cuvette in an Agilent 8453 spectrophotometer scanning from 380 to 780 nm. Specifically,

$$\text{Chromicity} = \frac{\int_{380\,\text{nm}}^{780\,\text{nm}} \text{LF}(1-T)}{\int_{380\,\text{nm}}^{780\,\text{nm}} \text{LF}} \quad (1)$$

where T = transmittance and LF = luminosity function. The specific luminosity function used in the present work is the Judd-Vos modified CIE 2° photopic luminosity curve (also known as CIE $V_M(l)$) [7].

Clarity

Clarity of growth media was assessed using an ISO 7027 [8] compliant LaMotte 2020i turbidity meter, which uses an 860 nm infrared light source to minimize error due to sample color. Triplicate samples were vortexed immediately prior to measurement in order to homogenize any undissolved solids. Instrument readings greater than 600 Formazin Nephelometric Units (FNU) were considered unreliable and are reported simply as ">600."

Calcium ion concentration

Calcium ion concentration in growth media was determined using an Orion 5-Star meter (Thermo Scientific, Beverly, MA, USA) with a calcium ion selective electrode.

Viscosity

The viscosity of autoclaved media was measured using a rotary viscometer (model LV2000, Cannon Instrument Company, State College, PA, USA) set up with a low viscosity spindle and a thermostated sample chamber.

Foaming

The foaming tendency of autoclaved media was determined using adaptations of two semi-standardized methods [9]. Aliquots (25 mL) of medium were added to a Waring Model 7010S blender (Conair, East Windsor, NJ, USA) fitted with a 100 mL cup. The medium was blended for 15 s at low speed and drained into a graduated cylinder for 45 s. At 1 min post-blending, the total volume of foam and liquid was measured. At 5 min the volume of just the liquid portion was measured. These values were used to calculate the properties % Foam Expansion (%FE) and % Foam Liquid Stability (%FLS). Specifically,

$$\%\text{FE} = \left(\frac{\text{total volume at 1 min}}{\text{initial liquid volume}}\right) \times 100\% \quad (2)$$

$$\%\text{FLS} = \left[1 - \left(\frac{\text{volume of liquid at 5 min}}{\text{initial liquid volume}}\right)\right] \times 100\% \quad (3)$$

Statistical analysis

Statistical analysis was performed using SAS 9.2 (SAS Institute, Cary, NC, USA). Although all experiments were performed on all 18 experimental and 3 commercial peptones with multiple repetitions, in many cases results from similar peptones (such as 4, 8 and 16 h alkali hydrolysates of MBM) were grouped when they gave very similar results, in order to reduce the volume of data presented and to aid in interpretation. In these cases, root mean square error from the ANOVA analysis of the experiment was used as an estimate of pooled error for the overall experiment.

Results

Hygroscopicity

When stored in a very humid environment, all dry peptone powders exhibited high hygroscopicity (Fig. 1). For all samples, the majority of the mass gain occurred in the first 7 days of storage, but the samples continued to slowly gain mass for weeks (data not shown); the samples reached a constant mass by 66 days of humid storage. Enzyme-hydrolyzed peptones were generally more hygroscopic than their alkali-hydrolyzed counterparts. Although the average moisture absorption of the commercial peptones was comparable to that of the enzyme-hydrolyzed peptones, this is misleading; casamino acids absorbed 0.71 g water/g ($\sigma = 0.05$, $n = 3$), the highest observed for any sample type, while bactotryptone and bacto yeast extract on average absorbed only 0.33 g water/g ($\sigma = 0.03$, $n = 6$).

At the end of the experiment, no peptone remained as a free-flowing powder. The only samples that remained as apparently dry solids were those that were produced though the alkaline hydrolysis of blood meal. In these samples, the original powder condensed into hard chunks. All of the remaining samples went through more extensive physical changes resulting in forms ranging from wet, spongy masses to syrupy liquids.

Chromicity

The commercial peptones tested imparted a pale but easily perceptible yellow color to media they were used in (Fig. 2). Alkali-hydrolyzed peptones imparted slightly more color of roughly the same hue, but media made with either commercial or alkali-hydrolyzed experimental peptones appeared similar to the human eye. Media made with enzyme-hydrolyzed experimental peptones, on the other hand, had significantly different coloration. The hue of each medium was clearly correlated with the raw material the peptone was made from, and the coloring was much more intense than that of media made with commercial peptone. Media made with enzyme-hydrolyzed blood meal appeared black (blood meal itself appears black, not red).

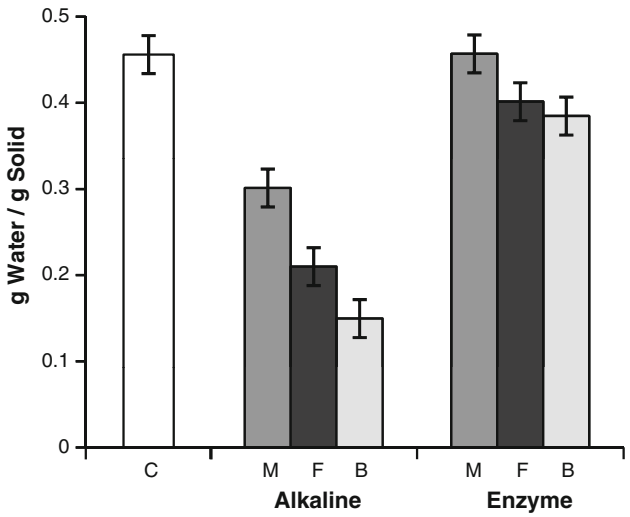

Fig. 1 Absorption of moisture by dry peptones stored in a humid environment. *M*, *F*, *B* and *C* indicate meat and bone meal, feather meal, blood meal and commercial peptone, respectively. *Error bars* represent $\pm\sqrt{\text{MSE}}$ (an estimate of pooled error) with 40 degrees of freedom (*df*)

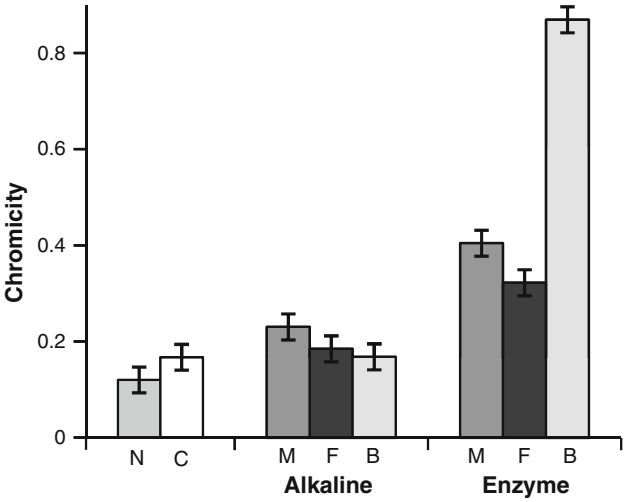

Fig. 2 Chromicities of fungal media made with experimental and commercial peptones. *N* indicates fungal media with no added peptone. *M*, *F*, *B* and *C* indicate meat and bone meal, feather meal, blood meal and commercial peptone, respectively. *Error bars* represent $\pm\sqrt{\text{MSE}}$ (an estimate of pooled error), 28 *df*

Clarity/autoclave stability

Commercial and experimental peptones were used to prepare two types of media, which will be referred to as the "fungal medium" and the "bacterial medium" for brevity. All media made with the three commercial peptones appeared very clear to the unaided eye. Turbimeter measurements showed that the bacterial medium became significantly hazier upon autoclaving; this was true also for a control bacterial medium that did not contain any peptone.

Most media made with enzyme-hydrolyzed peptones were moderately hazy both before and after autoclaving, with the haze increasing to some extent after autoclaving (Fig. 3, only post-autoclave data shown). The exception to this pattern was that fungal media made with blood meal peptones were very hazy after autoclaving.

Fungal media made with alkaline hydrolyzed peptones all appeared very clear, becoming significantly (but imperceptibly) less hazy upon autoclaving. Experimental

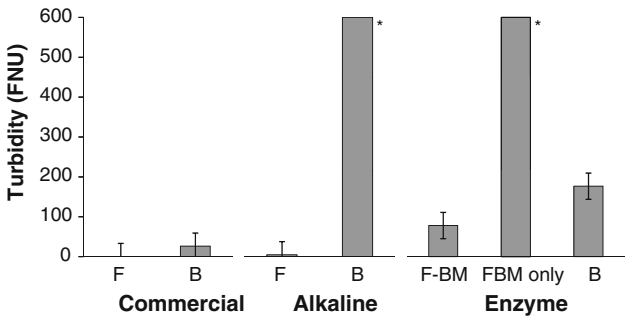

Fig. 3 The turbidities of fungal (*F*) or bacterial (*B*) media prepared with commercial peptones, or alkali- or enzyme-hydrolyzed experimental peptones, after autoclaving. In the enzyme portion, "BM only" indicates that only peptones made from blood meal are included, and "-BM" indicates that only peptones not made from blood meal are included. "*" indicates a reading of greater than 600 FNU, the highest turbidity that could be measured reliably. *Error bars* represent $\pm \sqrt{\text{MSE}}$ (an estimate of pooled error), 44 *df*

peptones made from a particular type of rendered protein decreased in haziness the longer they were hydrolyzed (Fig. 4).

In contrast, bacterial media made with alkali-hydrolyzed peptones all appeared quite hazy, and became hazy to the point of opacity when autoclaved. When this autoclaved media was quiescent, the solid particles causing the haze would settle, leaving behind a very clear-appearing liquid. These were the only cases in which the haze-producing particles settled.

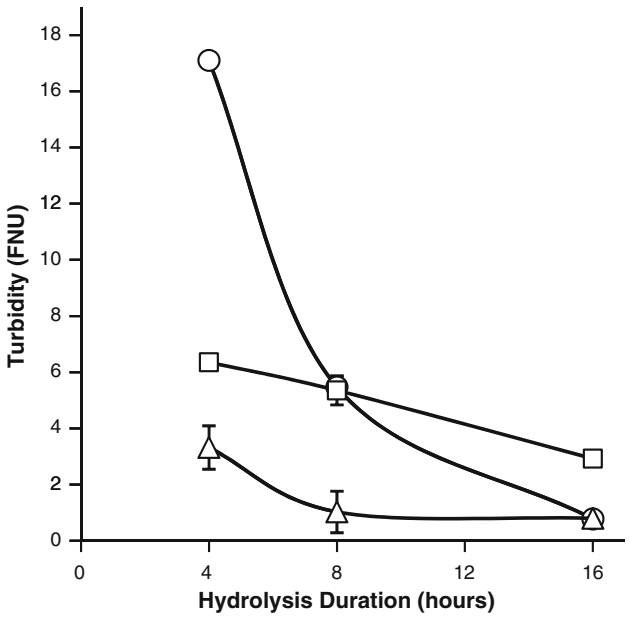

Fig. 4 The turbidity of autoclaved fungal media produced with alkali-hydrolyzed peptones. Rendered proteins including MBM (*circles*), FM (*squares*) and BM (*triangles*) were hydrolyzed for 4, 8 or 16 h. *Curves connecting points* are for illustration only. *Error bars* are present for all points and represent ± 1 sd

Some simplified media solutions were prepared in order to isolate the sources of haze. Both enzyme and alkali-hydrolyzed peptones made from meat and bone meal were dissolved in either water, 85 mM sodium phosphate buffer, or 7.5 mM ammonium sulfate, and then autoclaved. The concentrations of phosphate buffer and ammonium sulfate were equivalent to those used in the bacterial medium. Precipitation and very high turbidity were only produced in the media containing alkaline hydrolyzed peptone and phosphate buffer (Fig. 5a); the moderate turbidity of the enzyme-hydrolyzed peptone solutions was little affected by the inclusion of either additive. Analysis of the solution of alkali-hydrolyzed peptone and water shows that this peptone contributed calcium ions to the solution; the enzyme-hydrolyzed peptone did not contribute a measurable concentration of calcium ions (Fig. 5b). The solution of alkali-hydrolyzed peptone with added phosphate buffer had a calcium ion concentration that was too low to measure, suggesting that the calcium had participated in the formation of the precipitate.

Viscosity

No experimental or commercial peptone had a significant effect on the viscosity of the medium it was used in (Fig. 6).

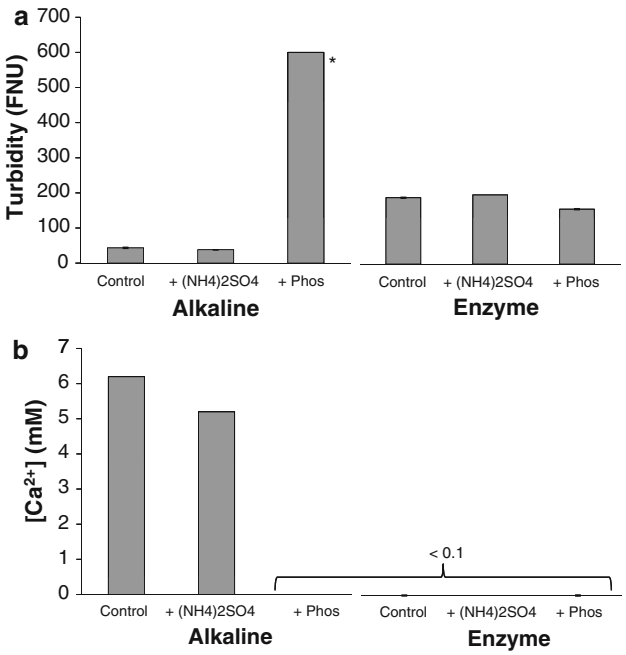

Fig. 5a–b The **a** turbidities and **b** calcium ion concentrations of simplified media. The alkali- or enzyme-hydrolyzed peptones used were made from MBM. "*" indicates a reading of greater than 600 FNU, the highest turbidity that could be measured reliably. *Error bars* are present for all points and represent ± 1 sd

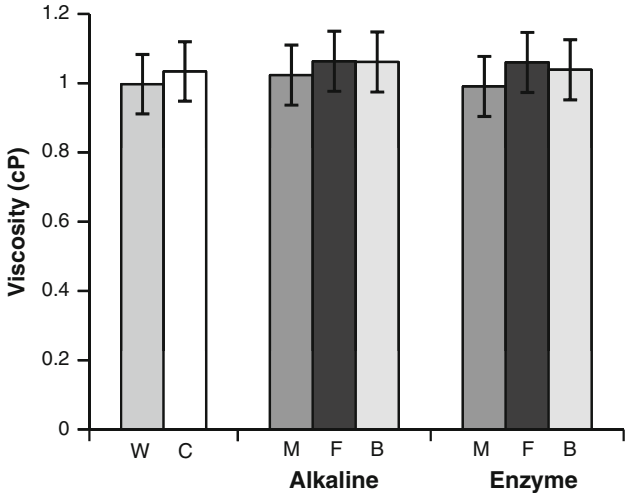

Fig. 6 Viscosities of fungal media made with experimental and commercial peptones. *M*, *F*, *B* and *C* indicate meat and bone meal, feather meal, blood meal and commercial peptone, respectively. *Error bars* represent ± $\sqrt{}$MSE (an estimate of pooled error), 44 *df*

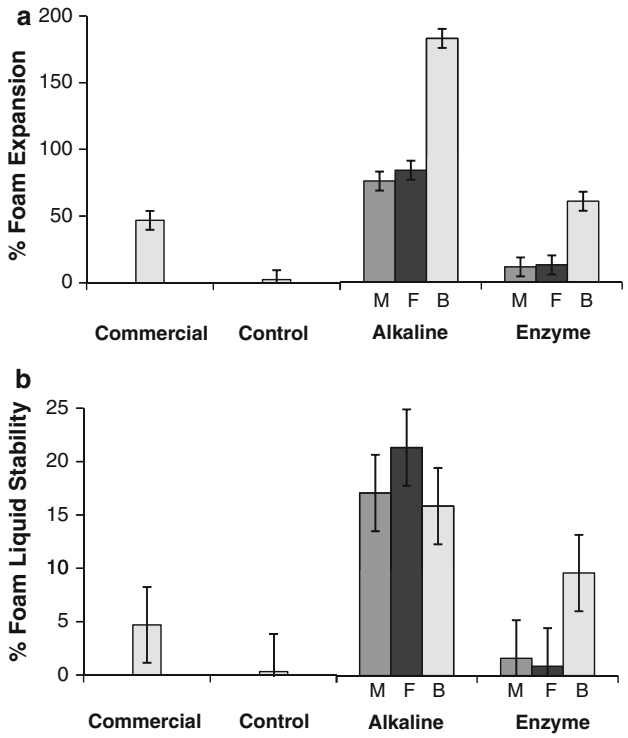

Fig. 7a–b Percent **a** foam expansion and **b** foam liquid stability. *M*, *F* and *B* indicate meat and bone meal, feather meal and blood meal, respectively. *Error bars* represent ± $\sqrt{}$MSE (an estimate of pooled error), 43 *df*

Foaming

Control media, made without any peptone, displayed very little tendency to foam when mixed with air (Fig. 7a), and what foam did form was very unstable and quickly dissipated (Fig. 7b). All peptones tested, both commercial and experimental, promoted the formation and stabilization of foam when used in media. Media made with alkaline hydrolyzed peptones consistently produced larger volumes of more stable foam than either commercial or enzyme-hydrolyzed peptones. Enzyme-hydrolyzed peptones other than those made from BM produced only very small amounts of unstable foam.

Discussion

Hygroscopicity

The experimental peptones were all found to be highly hygroscopic, but not outside of the range observed for commercial peptones. The overall greater moisture absorption of the enzyme-hydrolyzed peptones, compared to alkali-hydrolyzed peptones, may be partly explained by differences in the average molar masses of the two types of peptones. The molar mass distributions of the experimental peptones are explored in detail in a separate publication (in preparation), and a representative portion of these results is reproduced in Table 3. The enzyme-hydrolyzed peptones tended to have lower average molar masses; in a peptone, lower molar mass corresponds to more free carboxylic acid and amino functional groups per gram, and consequently more sites for forming hydrogen bonds with water molecules. This logic, however, does not explain the relatively low moisture absorption of bacto tryptone and yeast extract, which also have very low average molar masses. It is possible that during production these two commercial peptones are dehydrated in some manner that renders them resistant to rehydration.

Some samples went through greater physical changes than others. In an industrial context, however, all samples, including the commercial peptones, were too hygroscopic

Table 3 Number-average molar masses (Da) of hydrolysates

	Hydrolysis method	
	Alkaline	Enzymatic
Experimental		
MBM	320	285
FM	518	264
BM	676	242
Commercial		
BT	331	
BYE	113	
CA	238	

Specific data for alkali-hydrolyzed peptones come from analyzing samples hydrolyzed for 8 h; those for enzyme-hydrolyzed peptones come from analyzing samples hydrolyzed by Alcalase followed by Flavourzyme

to handle as powders without processing equipment specially designed to exclude moisture. Even with a fraction of the moisture absorption observed here, these sticky powders are very likely to cake during bin storage and clog pneumatic conveyance tubes.

Chromicity

The ideal microbial medium is colorless. Color in the medium can be carried over to the fermentation product and increase downstream purification costs. Even if the presence of color does not actually increase costs, operators are likely to regard an increase in the color of their medium unfavorably.

The property of *chromicity* is introduced here to quantify how close an experimental medium is to the color-free ideal, regardless of the color's hue. This property is particularly useful in the present research, because protein hydrolysates made from different raw materials have different hues, so measurement of the absorbance of each at any particular wavelength fails to provide a direct comparison of how colored they appear. Further, the sensitivity of the human eye has a strong wavelength dependence; absorbances of the same magnitude at different wavelengths will not have the same effect on how colored the solution appears.

The property of chromicity describes how intensely colored a solution appears to the human eye regardless of which colors are present. It assumes that light passes from an illumination source, through the sample, along a 1 cm pathlength before observation, and that scattering is negligible. The definition of chromicity incorporates a luminosity function to account for the varying sensitivity of the human eye to different wavelengths in the visible spectrum.

The alkaline-hydrolyzed experimental peptones studied in the present work were more colored than the commercial peptones, but the difference was small enough that they might be considered functionally equivalent by fermentation operators. The enzyme-hydrolyzed peptones were clearly not equivalent to the commercial peptones in this respect. It is possible that colored compounds in the raw material are stable to protease digestion, but are labile under alkaline conditions. There is some support for this hypothesis in the literature; one group [10] accomplished decolorization of hemoglobin hydrolysate by the addition of magnesium oxide.

Clarity and autoclave stability

The three commercial peptones tested produced low-turbidity, precipitate-free media. Alkali-hydrolyzed peptones performed similarly when used in the fungal medium, and would probably be considered functionally equivalent in this respect when used in an industrial context. All other media produced with the experimental peptones had a haze that was easily perceptible to the naked eye.

Within the group of fungal media made with alkali-hydrolyzed peptone, the observed decrease in haze with increased hydrolysis time corresponds with decreasing molar mass (manuscript in preparation). This would suggest that smaller peptides are less likely to contribute to haze. This conjecture is contradicted by the observation that fungal media produced with enzyme-hydrolyzed peptones were relatively hazy, even though these peptones are comprised of peptides with a lower average molar mass. It is possible that the enzyme itself, which has a high molar mass, is responsible for the haze.

The extreme haze and precipitation resulting from the use of alkaline hydrolysates in bacterial media was investigated further to determine its cause. Two distinct hypotheses were considered. First, the bacterial medium contains ammonium sulfate, which is a powerful precipitant of proteins. Potentially, this ammonium sulfate was precipitating the larger peptides present in the peptone. In this scenario, the use of enzymatic hydrolysates in bacterial media did not result in precipitation because it comprised smaller peptides that resist precipitation. Alternatively, the precipitate could result from the combination of calcium ions from the peptone and phosphate ions in the bacterial medium to form an insoluble species [11]. In this scenario, the alkali-hydrolyzed peptone has a high residual calcium concentration from the lime used in the hydrolysis. The evidence (Fig. 5) was found to support only the latter hypothesis. This particular problem could presumably be avoided by either using a non-phosphate buffer in the medium or using a base other than lime to hydrolyze the peptone raw material.

Viscosity

Gelatin, a common thickening agent, is produced through the partial hydrolysis of collagen. Since MBM is very high in collagen [12], it seemed reasonable that media made with hydrolyzed MBM would have a significantly raised viscosity. This was not the case.

All experimental peptones were equivalent to commercial peptones with respect to viscosity effects.

Foaming

Foaming of growth media when it is mixed or sparged is generally undesirable. Our finding that the alkali-hydrolyzed peptones (comprising relatively large peptides) were more foam promoting than the enzyme-hydrolyzed peptones (comprising relatively small peptides) is consistent with the findings of others [13]. The cause of the

particularly strong foam promotion of hydrolysates made with blood meal is not clear; possibly blood meal has a greater proportion of cell membranes than the other materials, and the phospholipids from these membranes are foam promoting.

Conclusions

None of the experimental peptones tested were found to perform equivalently to commercial peptones in all respects, but a few came reasonably close to that benchmark. Enzyme-hydrolyzed peptones made from either MBM or FM were equivalent or better than commercial peptones in terms of foaming and viscosity; in terms of the other properties, the inferiority of their performance seems amenable to improvement through further development. In several cases, peptone made from BM performed particularly poorly, and consequently it does not seem well suited to this application.

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